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Review of Recent Evidence of Toxic Effects of Hexachlorophene

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ABSTRACT. The published data on the toxicity of hexachlorophene in animals and man are discussed. Studies performed in the author's laboratory including hexachlorophene blood level determinations in animals and man are also reviewed. Hexachlorophene can produce paralysis in rats, rabbits, cats, and pigs and blindness in sheep. Microscopic examination of the brains of rats and monkeys given repeated doses of hexachlorophene shows status spongiosus of the white matter and normal gray matter. Small amounts of hexachlorophene are absorbed through the skin of animals as well as man. Aside from the neurological effects of hexachlorophene, the chemical uncouples oxidative phosphorylation which may cause body temperature elevation.

Pediatrics, 51:391, 1973, HEXACHLOROPHENE, TOXIC EFFECTS IN ANIMALS AND MAN.

THE literature on the toxicity of hexachlorophene was recently reviewed by Gump[1] and also by Kimbrough.[2]

Toxicity studies with hexachlorophene were performed in Sherman strain rats in our laboratory.[3] The acute oral LD_{50} for adult male rats was 66 mg/kg, for females 56 mg/kg, and for weanlings 120 mg/kg. In a reproduction study we found that the survival of offspring was slightly reduced in the F_1 generation when the parent rats were fed 100 ppm (8 mg/kg/day) hexachlorophene in their diet. They were mated 54 and 166 days after onset of exposure. The rats were fed the treated diets also during mating, gestation, and lactation. A dietary level of 20 ppm (1.5 mg/kg/day) did not affect reproduction.

A very pronounced effect on the brain was produced in rats fed hexachlorophene at the rate of 25 mg/kg/day. This effect could be observed microscopically in adult rats after they had been fed the hexachlorophene-containing diets for 4 days. A slight, but significant 6.5% increase in the wet weight of the brain over that of the controls was noticed after 2 weeks of exposure to diets containing 500 ppm (an average of 25 mg/kg/day) of hexachlorophene. An 11% increase in the wet weight of the brain over that of the controls was found after 4 weeks' exposure. When 500 ppm of hexachlorophene[4] were fed for 14 weeks, the wet weight of the brain averaged 2.60 gm, while the brains of the controls averaged 1.99 gm, which represents an increase in wet weight of 30.6%. The final body weights of these poisoned rats averaged 247 gm and those of the controls averaged 322 gm. Light microscopic examination of the brains of rats exposed to 500 ppm of hexachlorophene in the diet showed highly vacuolated white matter (status spongiosus) throughout the entire brain and spinal cord. The gray matter was normal. Electron microscopic examination of the white matter revealed that the vacuoles were due to a split in the interperiod line of the myelin sheath which had dilated and was probably filled with fluid. Occasionally, strands of myelin and granular material were observed within the vacuoles. At a dietary concentration of 100 ppm (5 mg/kg/day for 14 weeks) only a few rats showed vacuolated areas of the white matter. These areas were small and focal instead of diffuse. The other organs of these animals were normal grossly and microscopically. Four-week-old weanling rats showed status spongiosus of the white matter of the brain after a single dose of 100 mg/kg of hexachlorophene. The hexachlorophene was given by stomach tube in peanut oil. The brain lesion was most pronounced 4 days after dosing.

When a 3% concentration of hexachlorophene in propylene glycol or a detergent solution was applied daily to the skin of the back of rats for 30 days at the dosage levels of 48, 24, 12, and 0 mg/kg, erythema and ulceration of the skin developed at the dosage levels of 48 and 24 mg/kg. At the dosage level of 48 mg/kg the brains of all rats

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TABLE I

Hexachlorophene (HCP) Concentration
ppm in Blood
(ppm = μg HCP/g of whole blood)

	Infant		*Adult General Population*
	Cord *N = 50*	*Discharge* *N = 50*	*N = 14*
Range	0.003–0.102	0.009–0.646	<0.001–0.089
Mean	0.028	0.100	0.028
±S.E.	0.006	0.016	0.008

showed status spongiosus of the white matter. About half of the brains of the rats given 24 mg/kg/daily were affected. The brains of the rats with daily dermal exposure to 12 mg/kg were normal. The effect of hexachlorophene dissolved in propylene glycol was the same as when it was given in the detergent solution.

Monkeys have also shown this lesion. In a study conducted in another laboratory, newborn monkeys were washed daily with a 3% hexachlorophene solution in a detergent. They developed a diffuse status spongiosus of the white matter.[5] Santolucito[6] studied the effect of hexachlorophene in adult monkeys. He dosed squirrel monkeys each orally with 0, 1, 5, and 15 mg/kg/day for 28 days. The visual evoked potential was reduced in all exposed animals. The brains of these monkeys were essentially normal on microscopic examination. He then injected squirrel monkeys subcutaneously daily with 5 mg/kg of hexachlorophene for 37 days. The visual evoked potential in these animals was also reduced and a moderate diffuse status spongiosus of the white matter of the brain was observed. Brain tissue of the monkeys was also analyzed for hexachlorophene. Final hexachlorophene levels in the brain ranged from 0.18 ppm to 0.49 ppm. Monkeys that were given subcutaneous injections of 15 mg/kg/day for 1 week died. Their hexachlorophene brain levels were 0.8 ppm. The brains of the squirrel monkeys that died were not examined microscopically. (Recovery rate in brain was 75% in Santolucito's work).

Rats, rabbits,[7] pigs,[8] and cats (Dr. Stephen Krop, personal communication) show hindleg paralysis after exposure to large doses of hexachlorophene. In cats the pressure of the spinal fluid was increased. Sheep, according to Udall and Malone,[9] become blind. This blindness was irreversible while the paralysis in rats was reversible.[4] The brain lesion gradually resolves when rats are no longer exposed to hexachlorophene.[4] Other chemicals that produce status spongiosus of the white matter of the central nervous system in various species are triethyltin,[10] isoniazid,[11] and the halogenated benzanilide, 2-acetoxy-4-chloro-3,5-diiodobenzanilide.[12] Other halogenated salicylanilides may also cause this effect,[13] such as rafoxanide [3,5-diido-3′-chloro-4′-(p-chloro-phenoxy) salicylanilide].

The hexachlorophene levels in blood and various tissues of rabbits and rats that had been exposed to the chemical were also determined in our laboratory. We found in rabbits[14] that the peak hexachlorophene blood level was reached 7 hours following a single dermal application of 10 mg/kg hexachlorophene. Adult female rats that had been fed 100 ppm hexachlorophene in their diet for 258 days showed blood levels of 0.985–1.48 ppm with a mean of 1.21 ppm.[15] At this dietary level some rats demonstrated mild focal vacuolization of the white matter of the brain.

The blood levels of rats fed 500 ppm hexachlorophene in their diet for 55 days averaged about 8.5 ppm. When comparing blood levels it is important to know whether plasma or whole blood was analyzed for hexachlorophene. Flores and Buhler[16] found that in blood incubated with hexachlorophene 87% was bound to albumin in the blood of the newborn infant; 82% was bound to albumin in the blood of the rat. An additional significant amount was bound to the red blood cell membrane in both instances. Another important factor is the recovery rate. Our recovery rate for blood is now 95%. At the time we did the studies discussed below it was only 75%.

We determined hexachlorophene levels in 14 blood samples from the general population,[16] in 50 cord bloods and 50 blood samples from the same infants at the time of discharge from the hospital after birth.[17] Tables I and II give the results. The infants were washed daily with a 3% hexachlorophene-detergent solution. The number of washings and the interval after the last washing at the time the blood samples were obtained varied. The blood level determinations were done in collaboration with Drs. Nathenson and Finberg from the Albert Einstein College of Medicine.

These findings and those of Ulsamer, *el al.*[18] indicate that small amounts of hexachlorophene are absorbed through the intact human skin. This fact was also observed in rats by Carroll, *et al.*[19] who found that small amounts of radioactive hexachlorophene were absorbed through the intact skin of the tail of the rat.

When Curley and Hawk[15] studied the elimination of hexachlorophene from rats that had been fed 25 mg/kg/day for 55 days, they found that elimination is quite rapid at first. Half-life in kidney, liver, and blood is 10 hours. Half-life in the brain is about 19 hours. Excretion after a single dose follows the same pattern.

Caldwell, *et al.*[20] found hexachlorophene to be a very potent uncoupler of oxidative phosphorylation. Following a sublethal oral dose the maximal effect on oxidative phosphorylation occurred in the liver mitochondria about 12 to 15 hours after dosing and complete recovery required about 36 hours. Other well known uncouplers are pentachlorophenol and 2,4-dinitrophenols. Hexachlorophene was at least as or more effective. Cammer and Moore[21] also observed that hexachlorophene uncouples oxidative phosphorylation in brain and liver mitochondria. Hexachlorophene also inhibits cytochrome oxidase.[22,23] However, the inhibition of cytochrome oxidase seems to be of secondary importance. Strong binding of hexachlorophene to mitochondrial protein also occurs.[20]

A substantial increase in ATPase activity was evident in liver mitochondria. In rats receiving hexachlorophene orally (20 mg/kg) the ATPase activity of liver mitochondria isolated 18 hours later was three times that observed in control rats, but increasing the dose to 100 mg/kg did not result in a further ATPase increase.[20] Uncouplers of oxidative phosphorylation usually cause an elevation in body temperature. Pentachlorophenol is a good example of this. Nakaue, *et al.*[24] found that hexachlorophene elevated the body tem-

TABLE II

THE CONCENTRATION OF HEXACHLOROPHENE (ppm = μg/g) IN INFANT BLOOD AT BIRTH AND AT HOSPITAL DISCHARGE AFTER WASHINGS WITH HEXACHLOROPHENE SOLUTIONS

Infant Number	*Sex*	*Weight (gm)*	*Number of Washings*	*Concentration of Hexachlorophene*		
				Days in Hospital	*Cord Blood*	*Discharge Blood*
1	F	3,[illegible]	2	2	.010	.009
2	F	3,572	3*	2	.007	.010
3	F	3,[illegible]	3*	2	.007	.011
4	M	2,792	3	2	.011	.014
5	F	2,502	3	2	[illegible]	[illegible]
6	F	3,204	3	2	[illegible]	[illegible]
7	M	3,4[illegible]	4	2	.005	[illegible]
8	M	2,[illegible]	4	2	[illegible]	[illegible]
9	M	3,175	3	2	.007	[illegible]
10	F	2,4[illegible]	4	1	.010	[illegible]
11	F	3,[illegible]	5	3	.011	[illegible]
12	M	3,167	3*	2	[illegible]	.044
13	M	2,[illegible]	3	2	[illegible]	.045
14	F	2,948	3	2	[illegible]	.046
15	F	3,110	5	2	.005	.046
16	M	2,4[illegible]	3	2	.004	.048
17	M	3,175	3	2	.014	.049
18	M	3,175	3	3	[illegible]	[illegible]
19	M	2,657	3*	3	.011	.055
20	M	4,[illegible]	3	2	.100	[illegible]
21	M	3,317	4*	3	[illegible]	[illegible]
22	F	2,261	5	2	.007	.070
23	M	3,946	5	3	[illegible]	.071
24	M	2,4[illegible]	3*	2	.010	.072
25	M	2,041	7	3	[illegible]	.078
26	M	3,[illegible]	6*	7	[illegible]	[illegible]
27	F			2	[illegible]	.081
28	M	3,317	5	3	.111	.081
29	F	2,750	2*	2	[illegible]	.087
30	F	3,[illegible]	2	2	.017	[illegible]
31	F	2,[illegible]	3*	2	.014	[illegible]
32	F	3,4[illegible]	4*	3	<.000†	.100
33	M	3,657	3	2	.1[illegible]	.106
34	M	3,2[illegible]	3	2	[illegible]	.114
35	M	3,204	3	2	.010	.119
36	F	2,1[illegible]	3	2	[illegible]	.121
37	M	3,0[illegible]	3	2	.007	.121
38	F	2,337	4	3	.004	.12[illegible]
39	M	3,[illegible]	4	2	.013	.140
40	F	3,[illegible]	3	2	[illegible]	.151
41	M	3,[illegible]	3*	3	[illegible]	.16[illegible]
42	M	3,429	5*	3	.040	.170
43	F	3,0[illegible]	5	3	.020	.18[illegible]
44	M	2,[illegible]	4*	4	[illegible]	[illegible]
45	M	2,[illegible]	9	3	[illegible]	[illegible]
46	M	2,070	14	11	[illegible]	[illegible]
47	F	3,0[illegible]	4	3	[illegible]	[illegible]
48	M	3,0[illegible]	4	[illegible]	.010	[illegible]
49	M	4,0[illegible]	3	[illegible]	.041	[illegible]
50	M	3,907	5	2	.011	[illegible]

* Probable number of washes.

† None detected.

perature in rats. In animals poisoned with an uncoupling agent rigor mortis sets in very rapidly after death and is very pronounced. This has been described for pentachlorophenol and we have also observed it in rats poisoned with hexachlorophene.

REFERENCES

1. Gump, W. S.: Toxicological properties of hexachlorophene. J. Soc. Cosmetic Chemists 20:173, 1969.
2. Kimbrough, R. D.: Review of the toxicity of hexachlorophene. Arch. Environ. Health, 23:119, 1971.
3. Gaines, T. B., and Kimbrough, R. D.: The oral and dermal toxicity of hexachlorophene in rats. Toxicol. Appl. Pharmacol., 19(2):375, 1971.
4. Kimbrough, R. D., and Gaines, T. B.: Hexachlorophene effects on the rat brain: Studies of high doses by light and electron microscopy. Arch. Environ. Health, 23:114, 1971.
5. FDA Bulletin. Hexachlorophene and Newborns. Dec. 1971.
6. Santolucito, J. A.: Electroencephalograms and visual evoked potential of the squirrel monkey fed hexachlorophene. Tox. Appl. Pharm., 22(2):276, 1972.
7. Balazs, T.: Measurement of acute toxicity. *In* Paget, G. E., Ed.: Methods in Toxicology. Philadelphia; F.A. Davis Co., p. 60, 1970.
8. Larson, D. L.: Studies show hexachlorophene causes burn syndrome. Hospitals, J. Amer. Hosp. Ass., 42:63, 1968.
9. Udall, V., and Malone, J. C.: Optic nerve atrophy after drug treatment. *In:* The Problems of Species Difference and Statistics in Toxicology, Vol. XI. Amersterdam: Excerpta Medica Foundation, pp. 244-248, 1970.
10. Magee, P. N., Stoner, H. B., Barnes, J. M.: The experimental production of oedema in the central nervous system of the rat by triethyltin compounds. J. Path. Bact., 73:107, 1957.
11. Adornato, B., and Lampert, P.: Status spongiosus of nervous tissue. Acta Neuropath., 19:271, 1971.
12. Kurtz, S. M., Schardein, J. L., Fitzgerald, J. E., and Kaump, D. H.: Toxicologic studies with a halogenated salicylamide. Tox. Appl. Pharm., 14:81, 1969.
13. Brown, W. R., Rubin, L., Hite, M., and Zwickey, R. E.: Experimental papilledema in the dog induced by salicylamide. Tox. Appl. Pharm., 21:53[illegible], 1972.
14. Curley, A., Hawk, R. E., and Linder, R. E.: Hexachlorophene II. Percutaneous absorption in rabbits. Presented at the 161st National Meeting of the American Chemical Society, March 28-April 2, 1971, Los Angeles, California.
15. Curley, A., and Hawk, R. E.: Hexachlorophene I. Analysis in body fluids and tissues of experimental animals. Presented at the 161st National Meeting of the American Chemical Society, March 28-April 2, 1971, Los Angeles, California.
16. Flores, G., and Buhler, R.: In vitro hemolysis by chlorinated bisphenols. The role of hexachlorophene-protein interaction in the toxicity of chlorinated bisphenols. (Abst. 1725) Fed. Proc., 31:580, 1972.
17. Curley, A., Hawk, R. E., Kimbrough, R. D., Nathenson, G., and Finberg, L.: Dermal absorption of hexachlorophene in infants. Lancet, 2:296, 1971.
18. Ulsamer, A. G., Yoder, P. D., and Marzulli, F. N.: Determinations of hexachlorophene in human and experimental animal tissues. Tox. Appl. Pharm., 22(2):276, 1972.
19. Carroll, F. E., Salak, W. W., Howard, J. M., and Pairent, F. W.: Absorption of antimicrobial agents across experimental wounds. Surg. Gynec. Obstet., 125:974, 1967.
20. Caldwell, R. S., Nakaue, H. S., and Buhler, D. R.: Biochemical lesion in rat liver mitochondria induced by hexachlorophene [2,2'methylene bis (3,4,6-trichlorophenol)] Biochem. Pharm., in press.
21. Cammer, W., and Moore, C. L.: The effect of hexachlorophene on the respiration of brain and liver mitochondria. Biochem. Biophys. Res. Communications, 46:1887, 1972.
22. Gould, B. S., Frigerio, N. A., and Lebowitz, W. B.: The action of the bisphenolic compounds on succinoxidase, cytochrome c oxidase and lactic dehydrogenase of animal tissue. Archs. Biochem. Biophys., 58:476, 1955.
23. Nakaue, H. S., Dost, F. N., and Buhler, D. R.: Studies on the toxicity of hexachlorophene in the rat. In preparation.

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The 90-Dose LD$_{50}$ and a Chronicity Factor as Measures of Toxicity[1]

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The acute or 1-dose LD$_{50}$ is the universally accepted, primary way of expressing acute toxicity. Its numerical form permits useful comparisons between the acute toxicities of different compounds. There has been no generally accepted, comparable way to express the effects of repeated doses. This paper offers two proposals, a 90-dose LD$_{50}$ (or ED$_{50}$) and a chronicity factor, both of which may help to communicate the results of repeated doses. The form of the proposals may be new; the concepts on which they are based are well known to toxicologists.

The need for a brief test that will predict accurately the effect of dosing continued throughout life is recognized. It is also generally recognized that a 90-day test in rats represents a useful compromise between brevity and the lifetime of the animal. Weil and McCollister (1963) showed that the results of 90-day studies in rats and dogs were similar to corresponding lifetime studies in these species for a wide range of compounds. In our own laboratory, there was an indication that a 30-day test in the rat would be adequate for some compounds (e.g., potassium cyanide). However, a review of data on certain chemosterilants showed that, although 30 doses were entirely inadequate to reveal the potential injury caused by repeated doses of any of them, 90 doses gave for most of them essentially the same results as those of tests lasting twice as long. Although a test involving less than 90 doses would be adequate to reveal the effect of long-term exposure to many compounds, a 90-dose test is more generally valid for predicting long-term or chronic effects. Work under way at this laboratory shows that even a 90-dose schedule is inadequate to define the long-term toxicity of hempa; however, it was clearly evident after 90 or even somewhat fewer days that animals were still dying and that the exposure would have to be prolonged in order to assess the toxicity properly. Thus, partly because 90-dose tests are already widely accepted and in spite of recognized limitations, the first device suggested for reporting the results of repeated doses is called a 90-dose LD$_{50}$ (or 90-dose ED$_{50}$). It is statistically comparable to a 1-dose LD$_{50}$ (or 1-dose ED$_{50}$). In both, percent mortality (or other effect) expressed as probits is related to dose expressed as logarithms.

In calculating the conventional 1-dose LD$_{50}$, no account is taken of time although, of course, animals given a single dose do not respond to it simultaneously. The 90-dose LD$_{50}$ is managed in a similar way; the animals are held long enough after the last dose that any sick ones either die or recover. The two procedures differ since in the 1-dose test all animals in a group receive the same dosage, but in the 90-dose test animals in the same group may receive different total doses because some survive longer than others. This difference does not invalidate a comparison of the results of the two tests.

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[1] Presented at the Sixth Annual Meeting of the Society of Toxicology, Atlanta, Georgia, March 23–25, 1967.

In determining the acute oral LD$_{50}$, the compound is usually administered by stomach tube. It is proposed that in determining the oral 90-dose LD$_{50}$ (or ED$_{50}$), the compound be administered as a mixture in the diet. This difference in technique of oral administration introduces a second kind of difference (applying to this route only) between the two kinds of oral LD$_{50}$ value, but it has two advantages: convenience and realism. Obviously it is more convenient to maintain an animal on a special diet for 90 days than to dose the animal by stomach tube for the same period. It is also more realistic to administer repeated doses in the diet rather than by stomach tube, because if people receive relatively regular repeated doses of a compound, the intake is usually distributed throughout a considerable portion of the day as the result of either occupational or dietary exposure. On the contrary, if acute poisoning occurs in man, it is usually the result of a single massive exposure due to a splash or other spillage associated with occupation or the ingestion of a relatively concentrated material due to accident or suicide. Furthermore, if a number of compounds are administered by any particular route in such a way that the absorption of a single dose of each is concentrated in as short a period as possible but the absorption of repeated doses of each is distributed as evenly as is practical over each day, then any difference in cumulative effect of the compounds will be demonstrated to greatest advantage.

It is evident that the ratio between the 1-dose and 90-dose LD$_{50}$ values for a compound constitutes a measure of its cumulative effect. It is proposed, therefore, that the ratio, expressed as a quotient, be used in reporting the results of repeated dosing and that it be called the "chronicity factor." Since each factor is a ratio, the factors may be used to compare the tendency of different compounds to have cumulative effects without reference to their absolute toxicities.

If 90-dose LD$_{50}$ values are to be determined routinely, it would save time and money if a much briefer test could be found to predict the appropriate doses to use in the longer study. It was suggested by Scholz (1965) that death or other response of organisms receiving repeated exposures to a toxicant according to a regular schedule might form a straight line if the logarithm of dose were plotted against the logarithm of time necessary to produce the effect being measured. He offered two illustrations to indicate this was true. However, the longest dosing period involved in his illustrations was only 100 days or slightly less. For this and other reasons it is necessary to reconsider the suggestion of Scholz before deciding whether it offers a useful way of predicting the range of doses that should be tested to determine the 90-dose LD$_{50}$.

On general principles, it is evident that a complete curve of the kind described by Scholz would have 3 segments: (1) a first segment representing the minimal time necessary to produce an effect even with dosages larger than the minimal dosage required to produce it, (2) a second segment to represent the increasing times necessary to produce an effect with successively smaller dosages, and finally (3) a third segment indicating dosages that never produce the effect no matter for how long they may be continued.

METHODS

The animals used were adult Sherman strain white rats. During tests they were kept individually in Army Medical School type cages with a continuous supply of Purina Laboratory Chow[2] and water.

For determining its oral 1-dose LD_{50}, a compound was given to groups of rats by stomach tube at varying concentrations but at a constant volume (usually 0.005 ml/g of body weight) as described in detail by Gaines (1960).

To determine the oral 90-dose LD_{50} of a compound, appropriate concentrations in ground chow were fed to groups of rats for 90 days. All survivors were then fed uncontaminated feed for a minimum of 2 weeks, and if any of them were sick, they were observed for as long as necessary until they had died or recovered. The dose (expressed as mg/kg/day) was calculated from measured food consumption.

Both 1-dose and 90-dose LD_{50} values were calculated by the method of Litchfield and Wilcoxon (1949).

The chronicity factor for each compound was obtained by dividing its 1-dose LD_{50} (expressed as mg/kg) by its 90-dose LD_{50} (expressed as mg/kg/day). The resulting number is large (2.0 or more) for compounds that are relatively cumulative in their effects and small (less than 2.0) for compounds that show little cumulative effect. The factor of 2.0 is recognized as an arbitrary dividing point but it appears plausible from our limited data. In any event, if a compound were absolutely cumulative (in the sense that 1/90th of the 1-dose LD_{50} was exactly the 90-dose LD_{50}), the chronicity factor would be 90. A chronicity factor of 1.0 associated with oral intake indicates that daily ingestion of the 1-dose LD_{50} mixed into the regular diet leads to death of half of a very large population so exposed for 90 days.

The data obtained for the 90-dose LD_{50} of warfarin were used again in studying the 3 segments of a complete log-dose, log-time curve. This necessitated a slight deviation from the procedure just recommended for determination of the 90-dose LD_{50}, namely, the dose of warfarin that had produced no mortality during 90 days was continued for an additional 210 days in order to demonstrate the existence of the third segment of the curve.

In addition to tests carried out specifically for the purpose of exploring the concepts developed in this paper, the results of earlier studies from this laboratory were used to extend the range of compounds considered. The identity of these compounds is shown in Table 1.

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[2] Commercial sources and trade names are provided for identification only. Their mention does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

TABLE 1

IDENTITY OF COMPOUNDS MENTIONED IN THE TEXT

Common name	Chemical name
Apholate	2,2,4,4,6,6-Hexahydro-2,2,4,4,6,6-hexakis-(1-aziridinyl)-1,3,5,2,4,6-triazatriphosphorine
Azinphos-methyl	*O,O*-Dimethyl *S*-[4-oxo-1,2,3-benzotriazin-3(4*H*)-ylmethyl] phosphorodithioate
DDT	2,2-Bis(*p*-chlorophenyl)-1,1,1-trichloroethane
Dichlorvos	*O,O*-Dimethyl 2,2-dichlorovinyl phosphate
EPN	*O*-Ethyl *O*-*p*-nitrophenyl phenylphosphonothioate
Hempa	Hexamethylphosphoric triamide
Metepa	Tris[1-(2-methylaziridinyl)] phosphine oxide
Parathion	*O,O*-Diethyl *O*-(*p*-nitrophenyl) phosphorothioate
Warfarin	3-(α-Phenyl-β-acetylethyl)-4-hydroxycoumarin

RESULTS

The results of a 1-dose and a 90-dose LD_{50} study of warfarin are shown in Fig. 1. It may be seen that the curves are similar in slope although the dosages that proved critical differ by a factor of 20. Table 2 shows the LD_{50} values and related statistics for warfarin.

Table 3 summarizes the 1-dose and 90-dose LD_{50} values and also the chronicity factors for warfarin and several other compounds. The marked cumu-

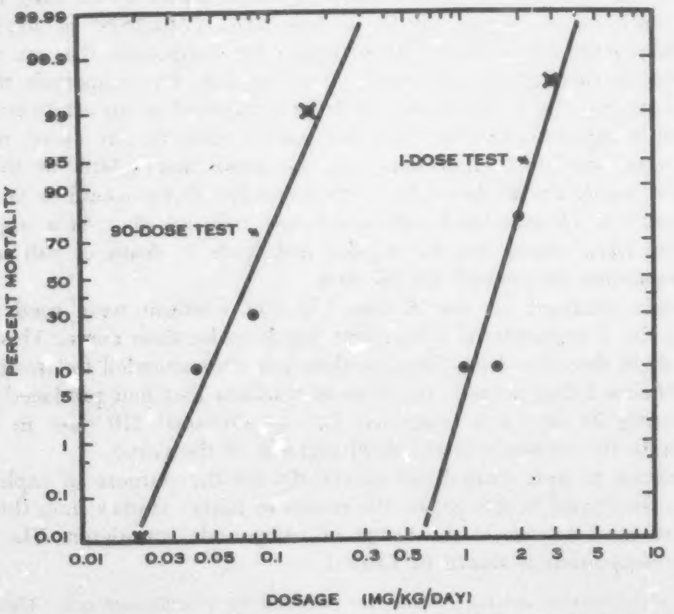

FIG. 1. One-dose and 90-dose oral LD_{50} curves for warfarin. Points adjusted [according to the method of Litchfield and Wilcoxon (1949)] are distinguished by a superimposed X.

TABLE 2

TOXICITY OF WARFARIN TO MALE RATS

Parameter	1-dose	90-dose
Number of rats tested	50	110
Survival time (days)	3–10	3–43^{a}
LD_{50}^{b}	1.6	0.077
19/20 Confidence limits^{b}	1.4–1.9	0.055–0.108
Lowest dose to kill^{b}	1.0	0.04^{c}
LD_{1}^{b}	0.84	0.032

[a] The true range may be 3–23 days; warfarin was probably not the cause of death in the rat that died after eating the compound for 43 days.

[b] Expressed as mg/kg for 1-dose test or mg/kg/day for 90-dose test.

[c] This death probably was not caused by warfarin. The smallest dosage to cause a death clearly related to warfarin was 0.08 mg/kg/day.

lative effect of warfarin and the chemosterilants, the small magnitude of such an effect of some organic phosphorus compounds, and the essential lack of cumulative effect of potassium cyanide are recorded. The 90-dose LD_{50} of warfarin was only 1/20 of the 1-dose LD_{50}, indicating a chronicity factor of 20 for that compound. It required daily ingestion of approximately a 1-dose LD_{50} of several organic phosphorus insecticides to kill half the test animals in 90 days, indicating a chronicity factor of approximately 1 in each instance. Rats tolerated daily 25 1-dose LD_{50}'s of potassium cyanide mixed with their regular food with no mortality, indicating a chronicity factor of <0.04. This tolerance for

TABLE 3

ABSOLUTE AND RELATIVE, ACUTE AND SUBACUTE ORAL TOXICITY OF CERTAIN COMPOUNDS TO RATS

Compound	Sex	1-dose LD_{50} (mg/kg)	90-dose LD_{50} (mg/kg/day)	Chronicity factor
Warfarin	M	1.6	0.077	20.8
Metepa	M	136^{a}	7.5^{a}	18.1
Apholate	M	98^{a}	17	5.8
DDT	M	113^{b}	>20	<5.6
Dichlorvos	F	56^{b}	>70^{c}	<0.80
Parathion	F	3.6^{b}	3.5	1.03
			3.1	1.16
Azinphos-methyl	F	11.0^{b}	10.5	1.05
EPN	F	7.7^{b}	12.0	0.64
Potassium cyanide	M	10	>250^{d}	<0.04

[a] From Gaines and Kimbrough (1964); both acute and long-term dosage of metepa was by stomach tube because of chemical evidence that the compound decomposed when mixed in food.

[b] From Gaines (1960).

[c] From Durham *et al.* (1957).

[d] No mortality occurred at 250 mg/kg/day, the highest dosage administered.

organic phosphorus compounds and cyanide undoubtedly indicates the ability of the body, and especially the liver, to detoxify these materials provided there is time in which to accomplish the task.

A replication of the 90-dose LD_{50} test for parathion produced results that were reasonably similar (Table 3). The second test was complicated by poor acceptance of the food. During the first week of that test, the consumption of food by the rats was so inhibited by the presence of parathion that their intake of poison was inversely proportional to the concentration of parathion in their diets. Acceptance of the higher concentrations gradually increased so that, with the exception of the highest dietary level, the average parathion intake for the entire test corresponded to the concentration of parathion in the diets.

Earlier study (Hayes and Gaines, 1959) suggested that the toxicity of warfarin might illustrate the 3 dose-response relationships characterized by the 3 segments of the log-dose, log-time curve outlined in the introductory section.

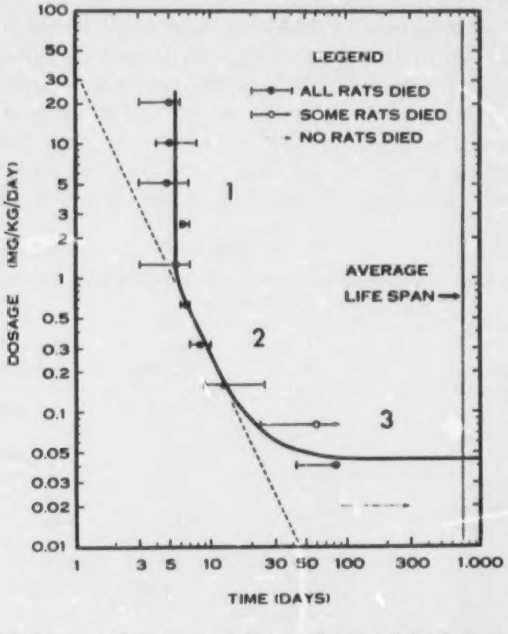

FIG. 2. Relationship between dosage of warfarin and time of death in rats. A short vertical line indicates the time of death of the first or last animal in a group to die, and a solid point indicates the geometrical mean time to death for a group in which all the animals died. In those groups in which some animals did not die, their survival is indicated by an arrow, and an open circle indicates the best estimated geometrical mean time to death. The tip of each arrow head indicates the end of the dosage period. Since it could not be assumed without proof that the survivors would live a normal life span, it was empirically assumed for purposes of calculation that the survivors died on the last day of the test. Thus, the true position of these estimated values always lies somewhere to the right of the open circle. Note that no rats died when dosed for over 300 days at a rate of 0.02 mg/kg/day.

The results of a new study to test this possibility are illustrated in Fig. 2. The 3 segments are well shown. The second segment was established by doses of 0.16, 0.32, 0.64, and 1.28 mg/kg/day, and in each instance the time necessary for half of the animals to die was 10 days or less. Thus, in the case of warfarin, the second segment was defined quickly. The test was satisfactory from the standpoint of brevity, one of the two requirements of a good predictive test. However, if one extrapolates the second segment of the curve (as shown in part by the dashed line in Fig. 2), one would predict that a dosage of approximately 0.002 mg/kg/day would kill half of a sufficiently large group of similar animals within 90 days. This prediction for warfarin is seriously inaccurate when compared to the 90-dose LD_{50} of 0.077 mg/kg/day based on all the dosage levels tested including a dose of 0.020 mg/kg/day, which was tolerated for more than 300 days without any mortality.

DISCUSSION

It may be concluded that the chronicity factor permits objective comparison of the cumulative effect of different classes of compounds and that it may permit comparison of the effects of different compounds of the same class. Whether these smaller intraclass distinctions are really significant or whether they are outweighed by differences caused by species or other factors must be determined by future experience.

It may be concluded also that by plotting the logarithm of time in days necessary to kill half of each group of animals against the logarithm of the daily dose necessary to produce this effect, one may predict the 90-dose LD_{50} of some compounds with acceptable accuracy but will predict only a much smaller, limiting value for other compounds. Probably the same is true for ED_{50} values. It would be fascinating to learn whether a chronic no-effect level could be predicted by dividing the one-dose no-effect level by the chronicity factor, but we have not explored this possibility.

Turning now to a discussion of other properties of log-dose, log-time curves, it is clearly evident that the first segment of the curve will be apparent only for those compounds that show an inherent delay in their action, and there is some reason to suppose that such compounds are necessarily cumulative. By an "inherent delay" is meant one not explained by the time required for absorption of the compound and its distribution to the target organ. Thus, an inherent delay is not fully overcome by substituting larger doses or by using intravenous injection. Examples are offered by (a) carcinogens, for which an induction period is apparently always required; (b) triorthocresyl phosphate, which produces paralysis in man or the chicken, but only following a delay of about 10 days; and (c) the coumarin-derivative anticoagulants, which inhibit prothrombin formation but have no clinical effect until the existing supply of prothrombin becomes depleted. A compound cannot produce a delayed effect unless it or its metabolites or a direct or indirect pharmacologic action persists until the effect appears. This persistence of a commond or its action is the very stuff of which cumulative effects consist. If the effect is sufficiently cumulative, the third segment of the theoretical curve may be lacking entirely. In fact, this was exactly what was found by Druckrey (1959) in connection with 4-dimethylaminoazobenzene (4-DAB); the appearance of tumors at sufficiently low dosage levels apparently was limited only because all the mice receiving those dosages died from old age before the first tumor had time to appear.

However, the presence of a delayed reaction following large doses does not exclude the possibility that small doses of the same compound may be tolerated. Figure 2 is an illustration of this kind of tolerance in rats fed warfarin. Another example is offered by the works of Siegel *et al.* (1965), who showed that a mixture of tricresyl and other triaryl phosphates, which produced paralysis of chickens and rabbits after only about 20 days of high-level exposure, was tolerated by both species for as much as 90 days when given at lower doses. Of course, it is easy to demonstrate tolerance for small dosages of most compounds, albeit they do not exhibit a significant latent period when absorbed at high dosages.

Thus, it appears that a few compounds (e.g., warfarin) exhibit all 3 segments of the theoretical curve, some (e.g., 4-DAB) exhibit the first and second segments only, and most compounds exhibit the second and third segments only. Presumably some compounds exhibit the second segment only, but no illustration is at hand. It is impossible to make a more exact statement at this stage because there has been so little study of comparative quantitative toxicology. In fact, it is not established that all compounds exhibit a typical second segment of the theoretical curve, although this appears likely.

As described earlier, rats showed poor acceptance of parathion mixed in their diet, especially during the first week of one test. This experience illustrated one of the difficulties of feeding tests, and the situation could be worse for other compounds. Another possible difficulty is spontaneous decomposition of some compounds when they are mixed in food. However, the possibility of such problems is inherent in feeding studies. If such problems occur, they are made no worse by an attempt to express the results quantitatively.

The real merit of reporting relevant toxicologic data in the form of (a) 90-dose LD_{50} or ED_{50} values and (b) chronicity factors must be determined by further experience. However, it must be pointed out that the merit of such reporting does not depend on use of a particular number of animals per group or on a particular method of calculating LD_{50} or ED_{50} values. Statistical methods that permit the use of a small number of animals per group and, therefore, an economical testing procedure have been suggested (Weil, 1952). An evaluation of such procedures as compared to the one used for the experiments reported in this paper is beyond the scope of this paper and irrelevant to the suggestion made in the following paragraph.

Since 90-day feeding tests are already widely used, it is suggested that—in addition to parameters conventionally reported—the results be reported in the form of 90-dose LD_{50} and ED_{50} values. By the use of these values and the chronicity factors calculated from them and the corresponding 1-dose LD_{50} and ED_{50} values, it may be hoped that the study of long-term toxicity may be made more quantitative.

SUMMARY

A 90-dose LD$_{50}$ (or ED$_{50}$) and a chronicity factor are proposed to help communicate the results of tests involving repeated doses of compounds. To determine the oral 90-dose LD$_{50}$, groups of animals are fed appropriate dietary levels of a compound for 90 days and then held long enough for any sick survivors to die or recover. Food consumption is recorded and doses are expressed as milligrams of compound per kilogram of body weight per day. Statistically, the 90-dose LD$_{50}$ is determined in the same way as the 1-dose LD$_{50}$ using logarithms of doses and percent mortality expressed as probits. A ratio of the 1-dose LD$_{50}$ and 90-dose LD$_{50}$ of a compound is a measure of its cumulative effects and is termed the chronicity factor. The largest one found so far is more than 500 times the smallest. These factors permit objective comparison of different classes of compounds, but whether the smaller distinctions within a class are significant, remains to be learned.

Since 90-day feeding tests are already widely used, it is suggested that—in addition to parameters conventionally reported—the results be reported in the form of 90-dose LD$_{50}$ and ED$_{50}$ values. By the use of these values and the chronicity factors calculated from them and from the corresponding 1-dose LD$_{50}$ and ED$_{50}$ values, it may be hoped that the study of long-term toxicity may be made more quantitative.

By plotting the logarithm of time in days necessary to kill half of each group of animals against the logarithm of the daily dose necessary to produce this effect, one may predict from a brief test the 90-dose LD$_{50}$ of some compounds with acceptable accuracy but only a much smaller, limiting value for other compounds. In addition to its limited predictive value, the log-time, log-dose relationship has considerable theoretical interest. The complete curve, exemplified by that for warfarin, shows 3 distinct segments corresponding to (1) an inherent delay in the action of the compound even when given at high dosages, (2) a gradual increase in the time necessary for intermediate dosages to produce their effect, and (3) the failure of sufficiently small dosages to produce an effect even when continued for the lifetime of the animal. Curves for many compounds lack 1 or even 2 of these segments.

REFERENCES

Druckrey, H. (1959). Pharmacological approach to carcinogenesis. *Ciba Found. Symp. Carcinogenesis: Mechanisms of Action, London, 1958*, pp. 110–130. Little, Brown, Boston, Massachusetts.

Durham, W. F., Gaines, T. B., McCauley, R. H., Jr., Sedlak, V. A., Mattson, A. M., and Hayes, W. J., Jr. (1957). Studies on the toxicity of *O,O*-dimethyl-2,2-dichlorovinyl phosphate. *A.M.A. Arch. Ind. Health* **15**, 340–349.

Gaines, T. B. (1960). The acute toxicity of pesticides to rats. *Toxicol. Appl. Pharmacol.* **2**, 88–99.

Gaines, T. B., and Kimbrough, R. D. (1964). Toxicity of metepa to rats. *Bull. World Health Organ.* **31**, 737–745.

Hayes, W. J., Jr., and Gaines, T. B. (1959). Laboratory studies of five anticoagulant rodenticides. *Public Health Rept.* **74**, 105–113.

Litchfield, J. J., and Wilcoxon, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exptl. Therap.* **96**, 99–133.

Scholz, J. (1965). Chronic toxicity testing. *Nature* **207**, 870–871.

Siegel, J., Rudolph, H. S., Getzkin, A. J., and Jones, R. A. (1965). Effects on experimental animals of long-term continuous inhalation of a triaryl phosphate hydraulic fluid. *Toxicol. Appl. Pharmacol.* **7**, 543–549.

Weil, C. S. (1952). Tables for convenient calculation of median-effective dose (LD$_{50}$ or ED$_{50}$) and instructions in their use. *Biometrics* **8**, 249–263.

Weil, C. S., and McCollister, D. D. (1963). Relationship between short- and long-term feeding studies in designing an effective toxicity test. *J. Agr. Food Chem.* **11**, 486–491.

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Effects of *o,p′*-DDT on the Adrenal Gland and Hepatic Microsomal Enzyme System in the Beagle Dog[1],[2]

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Effect of *o,p′*-DDT on the Adrenal Gland and Hepatic Microsomal Enzyme System in the Beagle Dog. COPELAND, M. F. AND CRANMER, M. F. (1974). *Toxicol. Appl. Pharmacol.* 27, 1–10. Four male beagle dogs received *o,p′*-DDT (50 mg/kg/day) in corn oil, administered in gelatin capsules, for 32 days. Four dogs used as controls received only corn oil. The treated dogs maintained body weights comparable to the controls and there were no overt signs of toxicity. The treated dogs showed a decreased eosinopenic response to ACTH after day 16. There was no significant difference in the plasma sodium and potassium concentrations or in the hematocrit before or after ACTH, nor in the 17-hydroxycorticosteroid (17-OHCS) response to ACTH between the treated and control dogs on any of the test days.

The activity of the following hepatic microsomal enzyme systems showed a significant increase: oxidative hydrolysis of EPN, epoxidase, oxidative desulfuration and detoxification of parathion, glucuronyltransferase, and azo-reductase. The *O*-demethylation of *p*-nitroanisole showed no change. The treated dogs had significantly larger livers and exhibited higher total protein, microsomal protein and cytochrome P-450 content.

There was no significant difference in the adrenal 17-OHCS concentrations between the treated and control dogs. The treated dogs had significantly larger adrenals. Histologic studies showed cells in the fasciculata of the treated dogs to be larger, more vacuolated, and devoid of acidophilic cytoplasm with hyperchromatic nuclei which frequently occupied a peripheral position in the cell. The zona glomerulosa, zona reticularis, and medulla showed no pathologic alterations.

A study of tissue distribution suggested that *o,p′*-DDT was rapidly converted to *o,p′*-DDD in the liver; however, the *o,p′*-DDD did not accumulate in the other tissues to the same extent as the *o,p′*-DDT.

These results suggest that *o,p′*-DDT does not block the synthesis or release of corticosteroids by the adrenal cortex but does stimulate the activity of the hepatic microsomal enzyme systems which are responsible for drug metabolism and the hydroxylation of steroid hormones in the beagle.

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[1] Presented in part at the Eleventh Annual Meeting of the Society of Toxicology, Williamsburg, Virginia, March, 1972.

[2] This research was part of a thesis submitted to the Graduate School of the University of Miami in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology.

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Cueto *et al.* (1958) presented data on the isolation, purification and identification of *o,p'*-DDD [1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane] from technical DDD and demonstrated an adrenocortical inhibitory effect of the *o,p'*-isomer in dogs. Nichols and Hennigar (1957) indicated that *o,p'*-DDD was one of the active principles present in technical DDD. Under specific conditions of dosage and time of administration, *o,p'*-DDD produced in dogs a selective atrophy of the inner zones of the adrenal cortex without significant effects on the outer glomerulosa (Nichols, 1961). This treatment produced an animal deficient in glucocorticoids but not mineralocorticoids.

In humans, the administration of *o,p'*-DDD for treatment of Cushing's syndrome reduced the clinical and biochemical signs before any effect was seen on the rate of cortisol secretion from the adrenal gland. There was a stimulatory effect on cortisol hydroxylation in man which resulted in an increased excretion of 6β-hydroxylation products and a decrease in the excretion of tetrahydrocortisol conjugates (Bledsoe *et al.*, 1964; Southren *et al.*, 1966a,b). The authors attributed the early effects of *o,p'*-DDD administration to the extra-adrenal metabolism of cortisol. Since the liver was considered to be the primary site for A-ring reduction and conjugation of cortisol as well as the 6β-hydroxylation of cortisol, it was suggested that *o,p'*-DDD may exert part of its action through its effects on the liver.

Hart and Fouts (1963) demonstrated that DDT stimulated the activity of the hepatic mixed function oxidase in rats. DDT has been shown to be a more potent stimulator of hepatic microsomal enzymes responsible for the metabolism of drugs and the hydroxylation of steroid hormones than DDD (Gillett *et al.*, 1966; Hart and Fouts, 1965).

Berndt *et al.* (1967) reported that the administration of *o,p'*-DDT [1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane] to dogs reduced the plasma concentrations of cortisol and corticosterone and their response to ACTH by 30–50%, while the administration of *p,p'*-DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] did not affect the plasma concentrations of cortisol and corticosterone and their response to ACTH. On the basis of this study they suggested that *o,p'*-DDT blocked the synthesis of corticosteroids in the adrenal and possibly stimulated their metabolism in the liver as did *o,p'*-DDD.

The present study was conducted to investigate the effect of oral administration of *o,p'*-DDT on adrenal function and hepatic mixed function oxidase systems in the beagle dog.

METHODS

Adult male beagle dogs weighing 11–14 kg (Hazelton Research Animals, Inc., Cumberland, Virginia) were maintained in outdoor runs and fed a diet of Commercial Purina Dog Chow mixed with Ken-L-Ration® canned dog food. During several months of adjustment to their environment the dogs were weighed periodically and their general appearance noted. Venipunctures were performed periodically to accustom the dogs to the technique. A preliminary experiment indicated the maximum response of plasma 17-hydroxycorticosteroids and circulating eosinophils to ACTH administration required 2 and 4 hr, respectively. Tests for adrenal cortical function were performed on all dogs to ensure normal functioning adrenals prior to administration of *o,p'*-DDT.

Four dogs were treated with o,p'-DDT[4] dissolved in corn oil, po, in gelatin capsules at a dose of 50 mg/kg/day, daily, for 32 days. Four dogs served as controls and received an equivalent amount of corn oil. The dogs were weighed weekly, and the dose was adjusted to weight change. The dogs were observed for overt clinical signs of toxicity.

Adrenal cortical function tests and analysis of blood for o,p'-DDT were performed on all dogs 2, 4, 8, 16 and 32 days after initiation of treatment.

On day 32 the animals were weighed and sacrificed; the liver and adrenal weights were recorded. Liver samples were homogenized, and hepatic enzyme activity was determined *in vitro*. Histologic examination was performed on the adrenals of all dogs. Adrenal 17-hydroxycorticosteroid (17-OHCS) concentrations were determined, as were total liver and microsomal protein content and hepatic cytochrome P-450 concentration.

At the time of sacrifice, samples of adrenal, liver, testis, and adipose tissue were analyzed by gas–liquid chromatography for o,p'-DDT and metabolites.

Evaluation of adrenal cortical function. The adrenal cortical function test in intact conscious dogs started at 8:00 am each day to eliminate the effect of diurnal variation and consisted of the following procedure: A sample of blood (10 ml) was withdrawn from the femoral vein of the dog. Ammonium heparin was used as an anticoagulant. ACTH,[5] 40 U, was then injected through the same needle into the femoral vein. Two hr later a second 10-ml sample of venous blood was obtained, and finally a 5-ml blood sample was taken 4 hr after the ACTH injection. Plasma 17-hydroxycorticosteroids were determined in the initial sample and in the 2-hr sample. An eosinophil count, plasma sodium and potassium concentrations and hematocrit were determined in the initial and in the 4-hr sample. Concentrations of o,p'-DDT and metabolites were determined in the initial blood sample by the method of Walker and Thompson (1971).

Eosinophils were counted by the method of Cueto and Brown (1958).

Plasma and adrenal hydroxycorticosteroid concentrations were determined by an adaptation of the method of Porter and Silber (Peterson *et al.*, 1955, 1957) with the exception that the volume of the final solution was reduced to 50 μl. This increased the sensitivity of the method to 0.04 μg of cortisol. Absorbance readings were made at 410 nm, and corrections for "extraneous chromogens" was made as described by Silber and Porter (1954).

Hematocrits were determined in duplicate using microhematocrit tubes and an International Microhematocrit Centrifuge. Plasma sodium and potassium concentrations were determined by flame photometry utilizing an internal lithium standard

The left adrenal of each dog was examined using routine hematoxylin and eosin-stained paraffin sections.

Determinations of hepatic enzyme activity. The enzyme systems investigated were mixed function oxidases [*O*-demethylation of *p*-nitroanisole and oxidative hydrolysis of *O*-ethyl-*O*-(4-nitrophenyl) phenylphosphonothioate (EPN), Kinoshita *et al.*, 1966], glucuronyltransferase (Chadwick *et al.*, 1971), epoxidase activity (Gillett *et al.*, 1966), azo-reductase activity (Fouts *et al.*, 1957) and the oxidative desulfuration and oxidative detoxification of parathion (Davis *et al.*, 1973).

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[4] 1-(*o*-Chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane (o,p'-DDT) was the pure (>99%) recrystallized isomer having a melting point of 73–75°C and was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

[5] Adrenocorticotropin (ACTH) was used as the commercially available H.P. Actar Gel obtained from Armour Pharmaceutical Company, Chicago, Illinois. 40 USP units were administered.

Protein and cytochrome P-450 determinations. Total liver and microsomal protein were determined by the procedure of Gornall *et al.* (1949). Hepatic cytochrome P-450 concentration was determined by the procedure of Alvares *et al.* (1968).

Tissue analysis by gas–liquid chromatography. Samples of liver, adrenal, testis and adipose tissue were analyzed immediately upon sacrifice for *o,p'*-DDT and metabolites by the method of Enos *et al.* (1967).

Statistics. All data was statistically evaluated using Student's *t* test.

RESULTS AND DISCUSSION

Oral administration of *o,p'*-DDT for 32 days produced no significant change in body weight (Fig. 1). There were no overt signs of toxicity observed among the dogs during the treatment period.

Adrenal Cortical Function

Results from this study demonstrate both histologically and physiologically that the zona glomerulosa of the adrenals in the treated dogs remained relatively intact. At the time of sacrifice the adrenal glands of the treated dogs were significantly larger ($p < 0.01$) than the control dogs (Fig. 1). There was no apparent adrenocortical atrophy in the H and E stained sections of the treated dogs. The zona glomerulosa was relatively intact. An indication of salt and water balance was obtained from the hematocrit, and plasma concentrations of sodium and potassium. There were no significant differences in any of these parameters on any of the test days, suggesting that treatment did not alter mineralocorticoid concentrations. Cueto and Moran (1968) did not detect any signs of mineralocorticoid deficiency in dogs receiving *o,p'*-DDD for 2 weeks.

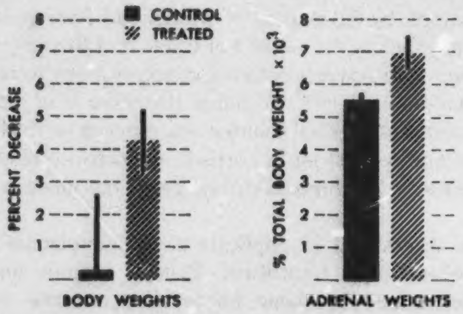

FIG. 1. Effects of *o,p'*-DDT on beagles. Dogs were treated with 50 mg/kg/day po for 32 days. Results represent the mean values ± SE for the 4 dogs in each group.

The zona fasciculata of the H and E stained sections showed occasional foci of large, vacuolated cells, devoid of acidophilic cytoplasm. The nuclei of the altered fasciculata cells were hyperchromatic and frequently occupied a peripheral position in the cell, in contrast to their central location in the normal cell. The reticularis and medulla appeared unchanged. These findings differ from the findings of Cueto and Moran (1968), who observed that dogs treated with DDD for 2 weeks had smaller adrenal glands than untreated dogs and that there was marked atrophy of the fasciculata and complete atrophy of the reticularis.

All methods for determining the adrenocortical inhibitory effect of a drug involves the observation of changes in adrenal function. Two acceptable tests with the required functional specificity are the increase in plasma 17-OHCS and the decrease in circulating eosinophils following the iv administration of ACTH. In *o,p′*-DDD treated dogs, plasma cortisol concentrations were low and the animals failed to respond to ACTH with a rise in plasma cortisol and fall in eosinophils (Cueto and Moran, 1968). Both of these tests were used to evaluate the effect of *o,p′*-DDT on the adrenal function in the beagle dog.

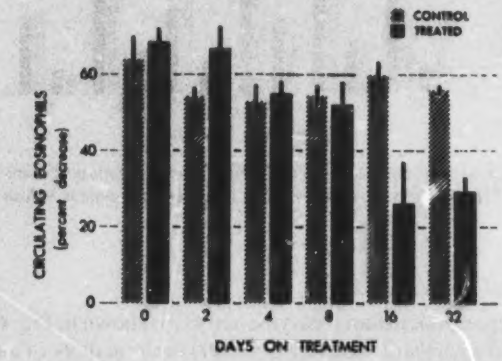

FIG. 2. Response of eosinophils 4 hr after the administration of ACTH, expressed as percent decrease from values prior to ACTH. Results represent the mean values ± SE for the 4 dogs in each group.

The eosinophilic response 4 hr after the administration of ACTH, expressed as the percent decrease of eosinophils from the values prior to ACTH, is presented in Fig. 2. A fall in eosinophils in the 4-hr sample of 50% or more from the level prior to ACTH injection was taken to indicate normal adrenal cortical function. There was no significant difference in the eosinopenic response to ACTH after 2, 4, and 8 days of treatment; however, the treated dogs showed a decreased eosinopenic response to ACTH after day 16 and day 32 of treatment.

Plasma concentrations of 17-OHCS, expressed as cortisol, before and 2 hr after ACTH stimulation of the adrenal cortex showed no significant difference on any of the test days (Fig. 3).

At the time of sacrifice a portion of the adrenals of each dog was analyzed for 17-OHCS concentrations. The results, expressed as ppm of cortisol, were 35.4 ± 6.4 in the control dogs and 22.4 ± 3.5 in the treated dogs (mean ± SE). There was no significant difference between the treated and control dogs.

Our findings differ from those of Berndt *et al.* (1967), who reported that treatment of dogs with 50 mg/kg/day *o,p′*-DDT for 7 days reduced the plasma concentrations of cortisol and the response to ACTH by about 30–50%.

The initial blood sample, on each test day, was analyzed for o,p'-DDT and metabolites. The amounts of o,p'-DDT in the control animals ranged from 0.017 to 0.023 ppm throughout the experiment, and the concentrations in the treated animals were 0.082 ppm after 2 days of treatment and increased to 0.604 ppm after 32 days of treatment. There was no o,p'-DDD detected in the blood on any of the test days.

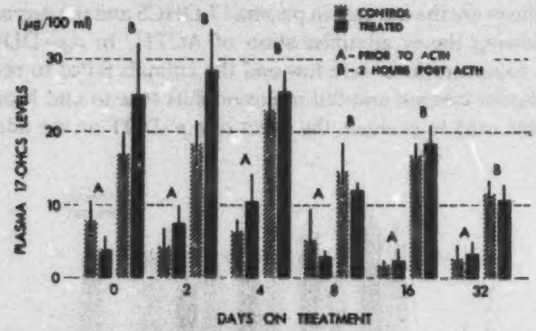

FIG. 3. Plasma 17-hydroxycorticosteroids (17-OHCS) concentrations before and 2 hr after the administration of ACTH, expressed as cortisol. Results represent the mean values ± SE for the 4 dogs in each group.

Hepatic Microsomal Enzyme Activity

Alterations of hepatic microsomal enzyme activity is shown in Fig. 4. Treatment with o,p'-DDT produced significant increases ($p < 0.01$) in the activity of all enzyme systems studied except *O*-demethylation of *p*-nitroanisole.

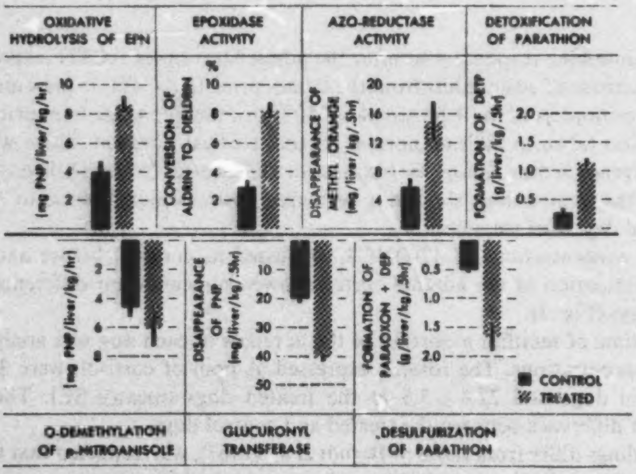

FIG. 4. o,p'-DDT-induced alterations of in vitro hepatic enzyme activity. Results represent the mean values ± SE for the 4 dogs in each group.

Liver weight, total protein, microsomal protein, and microsomal cytochrome P-450 concentration data are summarized in Fig. 5. The ratio of the liver weight to the body weight of the treated dogs was significantly larger ($p < 0.001$) than that of the control dogs. Treatment with o,p'-DDT also produced significant increases ($p < 0.01$) in total liver protein, microsomal protein, and microsomal cytochrome P-450 concentrations. Increases in these parameters frequently accompany stimulated microsomal enzyme activity. The results of this study indicate that treatment with o,p'-DDT stimulated the activity of various liver microsomal enzymes in the dog.

Detailed studies on the effects of liver microsomal enzyme inducers showed that compounds which stimulate drug-metabolizing enzymes also stimulate steroid hydroxylases in liver microsomes. This accelerated hydroxylation of steroid hormones *in vitro* by liver microsomal mixed function oxidases was paralleled *in vivo* by an accelerated metabolism of the steroid hormones and an altered physiological action of the steroids. A current review of this subject was presented by Conney (1967).

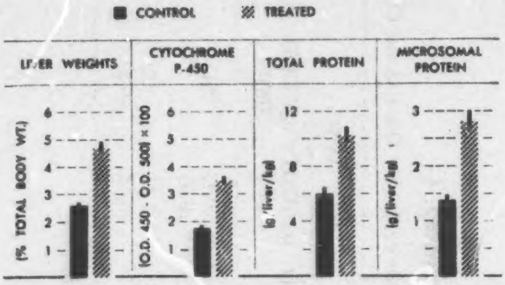

FIG. 5. Metabolic responses to o,p'-DDT in the beagle. Results represent the mean values ± SE for the 4 dogs in each group.

Kupfer *et al.* (1964) found that o,p'-DDD did not diminish cortisol formation, but increased the urinary excretion of polar (hydroxylated) cortisol metabolites in guinea pigs. Kupfer *et al.* (1969) also showed that activity of hepatic mixed function oxidase systems measured *in vitro* was enhanced by treatment with o,p'-DDD in the guinea pig. These findings suggest that o,p'-DDD stimulate cortisol hydroxylation, possibly by inducing the hepatic mixed function oxidase system.

Tissue Distribution and Storage

The results of gas–liquid chromatographic analysis of o,p'-DDT and metabolites in selected tissues are summarized in Table 1. The 1-to-1 ratio of o,p'-DDT to o,p'-DDD in the liver suggest that o,p'-DDT was rapidly converted to o,p'-DDD. However, the o,p'-DDD was not stored in the other tissues analyzed to the same extent as o,p'-DDT as shown by the 10-to-1 ratio of o,p'-DDT to o,p'-DDD in the adrenals, testis and adipose tissue. Of the tissues analyzed only adipose tissue stored greater quantities of o,p'-DDT than the adrenal gland. At the time of sacrifice the average amount of o,p'-DDT in the adrenals was 9.9 ± 1.8 ppm (mean ± SE). Cueto *et al.* (1958) reported average adrenal concentrations of o,p'-DDD to be 487 ppm after 14 days of treatment with o,p'-DDD.

TABLE 1

STORAGE OF o,p'-DDT AND METABOLITES IN TISSUES OF BEAGLE DOGS AT THE CONCLUSION OF THE EXPERIMENT[a]

Group	Dosage (mg/kg/day)	Days on treatment	Adrenals		Liver		Testis		Fat	
			o,p'-DDT (ppm)	o,p'-DDD (ppm)	o,p'-DDT (ppm)	o,p'-DDD (ppm)	o,p'-DDT (ppm)	o,p'-DDD (ppm)	o,p'-DDT (ppm)	o,p'-DDD (ppm)
Control	0	32	0.07 ±0.01	ND[b]	0.12 ±0.05	ND	0.10 ±0.04	ND	4.43 ±2.53	2.69[c] ±0.02
Treated	50	32	9.99 ±1.76	1.02 ±0.23	1.25 ±0.45	1.01 ±0.22	1.07 ±0.22	0.16 ±0.02	208.6 ±54.0	20.3 ±5.4

[a] Results represent the mean values ± SE for the 4 dogs in each group.

[b] ND = none detected.

[c] o,p'-DDD found in only 2 of the 4 control dogs.

The difference in action of *o,p′*-DDT noted in this study from the reported action of *o,p′*-DDD in the dog may be due in part to: (1) The difference in the accumulation of the two compounds in the target organ, the adrenal gland. (2) The fact that DDT is a more potent stimulator of liver microsomal enzymes than DDD.

Based on the results of this study, the following is postulated for the action of *o,p′*-DDT in the beagle: The chronic administration of *o,p′*-DDT stimulated the extra-adrenal metabolism of cortisol by liver microsomal enzymes which suggest an increased circulating concentration of 6β-hydroxycortisol which is accompanied by a decreased circulating concentration of cortisol. 6β-Hydroxycortisol has been shown to be biologically inactive in the feedback mechanism which regulates the secretion of ACTH from the anterior pituitary. The decrease in plasma cortisol initiates the release of ACTH from the pituitary which in turn stimulates the inner zones, fasciculata and reticularis, of the adrenal cortex to synthesize and secrete more 17-OHCS to bring plasma cortisol concentrations back to normal. The continuous inhibition of the feedback mechanism which shuts off the release of ACTH from the pituitary by high plasma concentrations of 6β-hydroxycortisol as a result of stimulated liver microsomal enzymes leads to the continued stimulation of the adrenal gland resulting in hypertrophy of the gland. This is supported by the results showing that the *o,p′*-DDT-treated dogs had significantly larger adrenal glands. As the stimulation of the adrenal gland continues the cells of the zona fasciculata, which are responsible for the synthesis of glucocorticoids, becomes exhausted and thus could account for the decreased eosinopenic response in the treated dogs after day 16 of treatment as well as the histologic changes noted in the zona fasciculata.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Richard Linko in the dosing and sacrificing of the dogs and Anita Peoples for her advice and assistance in the determination of hepatic microsomal enzyme activity.

REFERENCES

Alvares, A. P., Schilling, G., Levin, W. and Kuntzman, R. (1968). Alterations of the microsomal hemoprotein by 3-methylcholanthrene: Effects of ethionine and actinomycin D. *J. Pharmacol. Exp. Ther.* **163**, 417–424.

Berndt, S., Nakagawa, A. and Schwabe, U. (1967). Die Speicherung von *p,p′*-DDT und *o,p′*-DDT in der Nebennier und Ihre Bedeutung fur der Stoffwechsel von Corticosteron und Cortisol. *Arch. Pharmakol. Exp. Pathol.* **256**, 383–396.

Bledsoe, T., Island, D. P., Ney, R. L. and Liddle, G. W. (1964). An effect of *o,p′*-DDD on the extra-adrenal metabolism of cortisol in man. *J. Clin. Endocrinol.* **24**, 1303–1311.

Chadwick, R. W., Cranmer, M. F. and Peoples, A. J. (1971). Comparative stimulation of γ-HCH metabolism by pretreatment of rats with γ-HCH, DDT, and DDT + γ-HCH. *Toxicol. Appl. Pharmacol.* **18**, 685–695.

Conney, A. H. (1967). Pharmacological implication of microsomal enzyme induction. *Pharmacol. Rev.* **19**, 317–366.

Cueto, C. and Brown, J. H. U. (1958). A rose Bengal-phloxine B stain for dog eosinophils. *Stain Technol.* **33**, 95–97.

Cueto, C. and Moran, N. C. (1968). The circulatory effects of catecholamines and ouabain in glucocorticoid-deficient animals. *J. Pharmacol. Exp. Ther.* **164**, 31–44.

Cueto, C., Brown, J. H. U. and Richardson, A. P., Jr. (1958). Biological studies on an adrenocorticolytic agent and the isolation of the active components. *Endocrinology* **62**, 334–339.

Davis, J. E., Cranmer, M. F. and Peoples, A. J. (1973). Gas-liquid chromatography of parathion metabolites as a sensitive index of induction of liver microsomal mixed-function oxidases. *Anal. Biochem.* **53**, 522–530.

Enos, H. F., Biros, F. J., Gardner, D. T. and Wood, J. P. (1967). Micro method for the determination of chlorinated pesticides in human tissue. Presented at the 154th meeting of the American Chemical Society, Chicago, Illinois, September 10–15.

Fouts, J. R., Kamm, J. H. and Brodie, B. B. (1957). Enzymatic reduction of Prontosil and other azo-dyes. *J. Pharmacol. Exp. Ther.* **120**, 291–300.

Gillett, J. W., Chan, T. M. and Terriere, L. C. (1966). Interactions between DDT analogs and microsomal epoxidase systems. *J. Agr. Food Chem.* **14**, 540–545.

Gornall, A. G., Bardawill, C. J. and David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751–766.

Hart, L. G. and Fouts, J. R. (1963). Effects of acute and chronic DDT administration of hepatic microsomal drug metabolism in the rat. *Proc. Soc. Exp. Biol. Med.* **114**, 388–392.

Hart, L. G. and Fouts, J. R. (1965). Further studies on the stimulation of hepatic microsomal drug-metabolizing enzymes by DDT and its analogs. *Arch. Exp. Pathol. Pharmacol.* **249**, 486–500.

Kinoshita, F. K., Frawley, J. P. and Dubois, K. P. (1966). Quantitative measurement of induction of hepatic microsomal enzymes by various dietary levels of DDT and toxaphene in rats. *Toxicol. Appl. Pharmacol.* **9**, 505–513.

Kupfer, D., Balazs, T. and Buyske, D. A. (1964). Stimulation by *o,p'*-DDD of cortisol metabolism in the guinea pig. *Life Sci.* **3**, 959–964.

Kupfer, D., Bruggeman, L. L. and Munsell, T. (1969). Studies on the occurrence of N-demethylase activity in adrenal and hepatic preparations from guinea pigs and rats: The effect of various substances on the magnitude of this activity. *Arch. Biochem. Biophys.* **129**, 189–195.

Nichols, J. (1961). Studies on an adrenal cortical inhibitor. In: *The Adrenal Cortex* (H. D. Moon, ed.). Harper (Hoeber), New York.

Nichols, J. and Hennigar, G. (1957). Studies on DDD. *Exp. Med. Surg.* **15**, 310–316.

Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B. and Bunim, J. J. (1955). The physiological disposition and metabolic fate of hydrocortisone in man. *J. Clin. Invest.* **34**, 1779–1794.

Peterson, R. E., Karrer, A. and Guerra, S. L. (1957). Evaluation of Silber-Porter procedure for determination of plasma hydrocortisone. *Anal. Chem.* **29**, 144–149.

Silber, R. H. and Porter, C. C. (1954). The determination of 17,21-dihydroxy-20-keto-steroids in urine and plasma. *J. Biol. Chem.* **210**, 923–932.

Southren, A. L., Tochimoto, S., Isurugi, K., Gordon, G. G., Kirkun, E. and Stypulkowski, W. (1966a). The effect of 2,2-bis(2-chlorophenyl-4-chlorophenyl)-1,1-dichloroethane (*o,p'*-DDD) on the metabolism of infused cortisol-7-^{3}H. *Steroids* **7**, 11–29.

Southren, A. L., Tochimoto, S., Stron, L., Ratuschini, A., Ross, H. and Gordon, G. G. (1966b). Remission in Cushing's Syndrome with o,p'-DDD. *J. Clin. Endocrinol.* **26**, 268–278.

Walker, A. C. and Thompson, J. F. (1971). A rapid procedure for the determination of organochlorine pesticide residues in blood serum. In: *Analysis of Pesticide Residues in Human and Environmental Samples* (J. F. Thompson, ed.), Section 5,A,(3),(a). Perrine Primate and Pesticide Effects Laboratory, Environmental Protection Agency, Perrine, Florida.

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ENVIRONMENTAL RESEARCH 6, 265–273 (1973)

The Ultrastructure of the Paraquat Lung Lesion in the Rat

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Adult female Sherman strain rats were fed 500 ppm paraquat (1,1-dimethyl-4,4-bipyridylium dichloride) in their diet. Four poisoned as well as three control rats were sacrificed 1, 3, 5, 7 and 11 wk after onset of exposure. Two well-developed paraquat lung lesions were encountered. The alveoli of these lesions contained a very prominent amorphous material which was highly osmiophilic when examined with the electron microscope. It consisted of concentrically arranged whorls which were surrounded by or next to less electrondense lattice-like material. Additional ultrastructural findings in those and other lungs consisted of edematous and vacuolated membranous pneumocytes (type I) as well as endothelial cells. The granular pneumocytes were increased in 3 of 20 lungs of exposed animals.

The dipyridylium compound paraquat (1,1-dimethyl-4,4-bipyridylium dichloride or dimethylsulfate) has a very selective effect on the lungs. This organ selectivity has so far not been satisfactorily explained. Several cases of human poisoning have been reported. Nienhaus and Ehrenfeld (1971) reported one of the most recent cases and also reviewed previous cases of human incidences of poisoning reported in the literature. Exposure in humans has usually been by ingestion, with the exception of one case where suicide was committed by injection (Almog and Tal, 1971). Early changes observed in the lung consisted of swelling and vacuolization of the cytoplasm of the membranous pneumocytes (Kimbrough and Gaines, 1970; Modie, Ivemark and Robertson, 1972; Nienhaus and Ehrenfeld, 1971; Smith, 1971; Toner *et al.*, 1970) and complete degeneration of these cells. Vijeyaratnam and Corrin (1971) found that rats surviving a single dose of paraquat for 14 days showed convoluted profiles of the basement membrane where the lining epithelium had completely disappeared. The underlying interstitium was widened by edema and showed fibrosis. In other parts slit-like alveoli lined only by a row of granular pneumocytes were seen. Brooks (1971) studied the effect of paraquat in the mouse lung. His work using electron microscopy showed early changes that consisted of sporadic focal endothelial cell swelling, blebbing and loosening of these cells from the underlying basal lamina. Damage to membranous (type I) pneumocytes also occurred early. In later stages exudate was present in alveolar air spaces and bronchiolar lumina. Fibroblasts increased in number, alveolar collapse was common, and proliferation of granular cells (type B) was noted. A thin osmiophilic lamella bordering alveolar epithelium in areas of lung collapse was also observed. Similar findings were reported by Smith (1971).

The present paper describes the ultrastructure of the well-developed lung

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lesion following chronic exposure to paraquat and adds additional findings to those already reported in the literature.

MATERIALS AND METHODS

Fifty adult female Sherman strain rats 4 mo old were used. They were divided into two groups of 30 and 20 rats according to a table of random numbers. Their food consumption was measured during the 1st, 4th, and 7th wk of exposure. The group of 30 rats was exposed to 500 ppm of paraquat as the dichloride salt in the diet while the remaining 20 rats were kept as controls. The poisoned diets were prepared by diluting the paraquat concentrate with ethanol. This solution was mixed into cornstarch and the solvent allowed to evaporate. The cornstarch mixture was then mixed into increasing amounts of ground chow to produce the desired concentration of paraquat. Four exposed rats and three control rats were sacrificed 1, 3, 5, 7, and 11 wk after onset of exposure. Lung tissue was fixed in buffered formalin and stained with hematoxylin and eosin for light microscopic

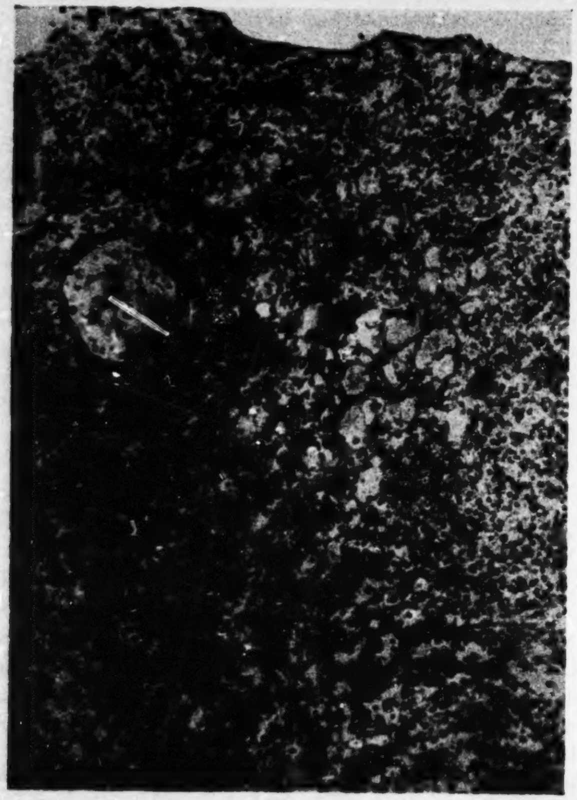

FIG. 1. Section of paraquat lung lesion as it appears under the light microscope. Note the light-staining amorphous material within the alveoli (arrow). Toluene blue, ×125 embedded in Maraglass.

study. For examination with the electron microscope the lung tissue was fixed in 5% phosphate buffered glutaraldehyde for 3 hr postfixed in 1% osmium tetraoxyde, embedded in Maraglass, stained with lead citrate and uranylacetate. The sections were examined under the Phillips EM 300 electron microscope. Tissue from Maraglass embedded blocks was also cut for the light microscope and stained with toluidine blue. The remaining rats that had not died were discarded.

RESULTS

Two rats died during the experiment. In three additional rats breathing was abnormal. The food consumption was slightly reduced during the first week of exposure to the poisoned diet. The average intake of paraquat during that week was 20.4 mg/kg/day, during the 4th and 7th week of exposure it was 29 and 29.6 mg/kg/day, respectively.

The lungs of all the control rats and the rats sacrificed 1 wk and 3 wk after onset of exposure were normal. After 5 wk on the paraquat poisoned diet, two rats had normal lungs, one showed several areas of consolidation in the lung on gross inspection and one showed ultrastructural changes only. The areas of consolidation contained proliferated epithelial cells surrounded by inflammatory cells and fibroblasts. When this tissue was embedded in Maraglass and stained with toluidine blue the alveoli contained a very prominent amorphous material (Fig. 1) which stains faintly pink with eosin. Oil red O stain of this material did

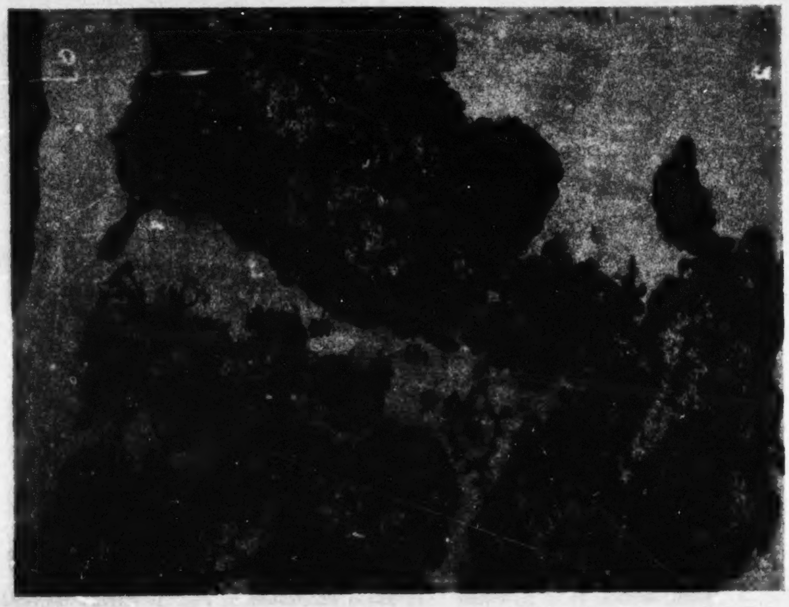

FIG. 2. Lung section illustrating the edematous endothelial cells. EN = endothelial cell. BM = basement membrane, EP = membranous pneumocyte. Lead citrate, uranylacetate, ×9,864.

not reveal any lipid. Electron microscopic examination of the area shown in Fig. 1 revealed again the fibroblasts, macrophages, and edematous endothelial cells (Fig. 2). In addition amorphous material in the alveoli was highly osmiophilic and consisted of concentrically arranged whorls. These whorls were next to or surrounded by less electrondense material which was arranged like a lattice. The material was very closely attached to the cell membrane (Figs. 3 and 4). Within occasional granular pneumocytes (type II) large whorls of the highly osmiophilic material were also seen. Many mitochondria in the granular pneumocytes and membranous pneumocytes showed signs of degeneration. The granular pneumocytes (type II) were increased in number, the membranous pneumocytes were vacuolated and an increase in collagen and widening of the basement membrane was seen (Fig. 5). The fourth lung which was normal on light microscopic examination showed a slight increase in interstitial collagen. The cytoplasm of the membranous pneumocytes contained occasional vacuoles. Occasional granular pneumocytes (type II) had less electrondense areas in the cytoplasm that were filled with a finely granular material (Fig. 6).

Seven weeks after onset of exposure four exposed and three control rats were sacrificed. The lungs of one rat were completely normal on light microscopic examination while the other three showed a few small focal areas of fibrosis. All four lungs showed ultrastructural changes. Vacuolated and edematous membranous pneumocytes were observed in the lungs of all 4 rats. The lungs of two

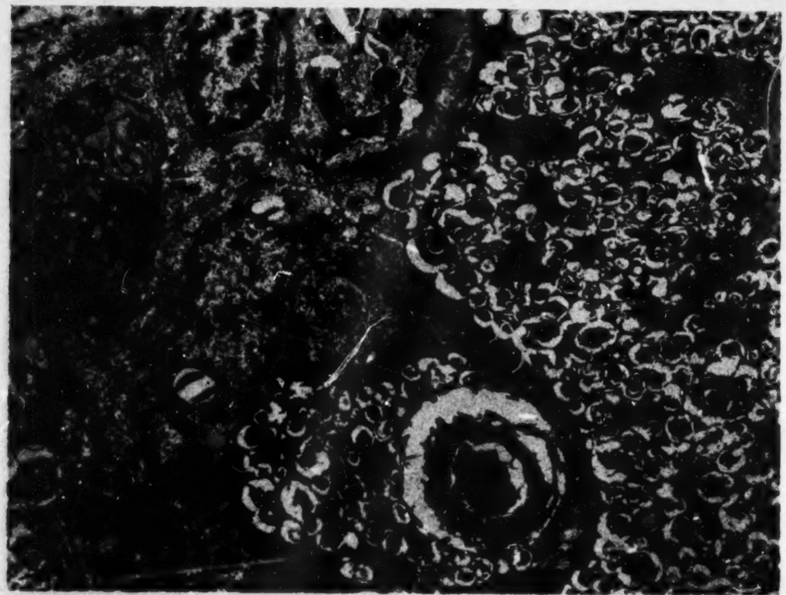

FIG. 3. Lung section from paraquat lung lesion illustrated in Fig. 1. Highly osmiophilic material forming concentrically arranged whorls is seen in the alveolar lumen and in the cytoplasm of granular (type II) pneumocytes. Adjacent to the whorls less electron-dense lattice-like structures are present. Lead citrate, uranyl acetate, ×4724.

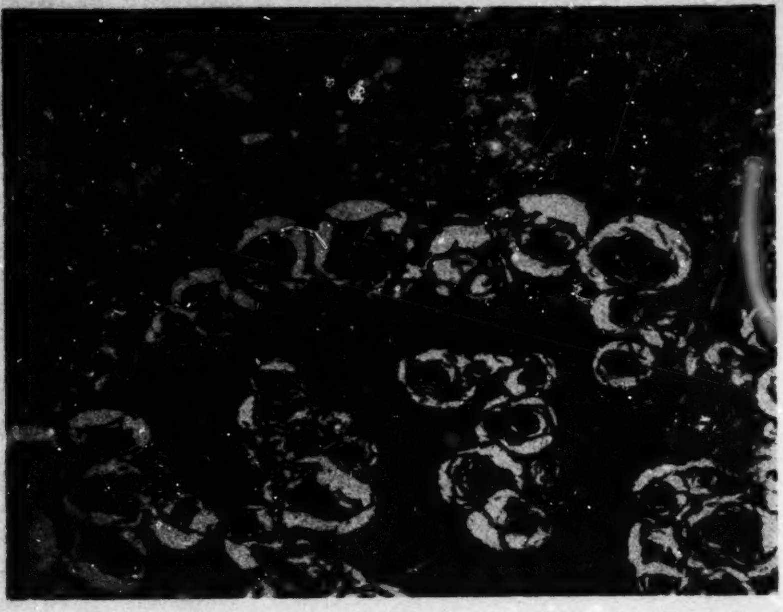

FIG. 4. Higher magnification of an area adjacent to that illustrated in Fig. 3 shows the close attachment of this material to the cell wall. Edema of the endothelial cells, an increase in collagen and elastin can also be observed. Lead citrate, uranyl acetate, ×9,864.

rats contained a great deal of surfactant, a material of laminated or striated structure within the alveoli (Fig. 7). The granular pneumocytes (type II) were more numerous in one of the lungs. In other areas the alveolar cells were completely destroyed and replaced by collagen and reticulum fibers. The lungs of this rat showed small areas of fibrosis under the light microscope.

Eleven weeks after exposure the lungs of one rat showed extensive consilidation at autopsy while the other three were normal. Light microscopic examination showed normal lung in two instances, and foci of hemorrhage and brown pigment in macrophages in one instance. The lungs of the fourth rat showed extensive areas of fibrosis, many macrophages contained a brown pigment. The alveolar epithelium had proliferated, was cuboidal and at times stratified.

Electron microscopic examination revealed normal lung in one rat. The lungs of the other rat that showed no light microscopic changes were found to contain occasional vacuolated membranous pneumocytes with degenerated mitochondria. A slight increase in surfactant was also observed. Similar but more pronounced findings were made in the rat that showed hemorrhagic areas under the light microscope. In this animal an increase in the collagen and reticulum in the basement membrane was observed. The ultrastructural changes of the animal showing a lung lesion grossly consisted of proliferation of granular pneumocytes, obliteration of alveolar lumina, edematous and vacuolated membranous pneumocytes, edematous endothelial cells (Fig. 2) and accumulation of highly osmio-

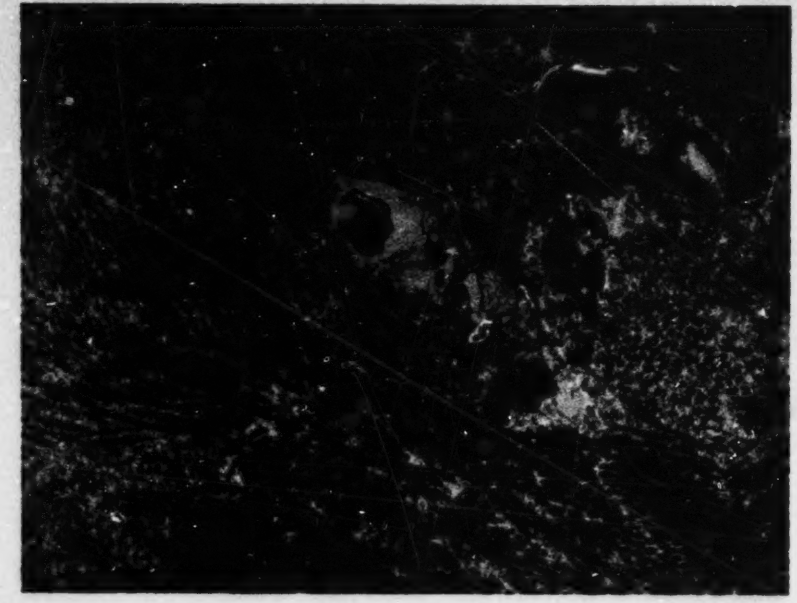

FIG. 5. Section from the paraquat lung lesion shown in Fig. 1 illustrating the pronounced increase in collagen. Lead citrate, uranyl acetate ×14,633.

philic material within the alveoli. This material consisted again of concentrically arranged whorls, which were next to or surrounded by a lattice of electrondense material (Fig. 4). Since the findings made eleven weeks after exposure were similar to those made earlier, the remaining animals in the study were discarded.

DISCUSSION

This study confirmed again that the primary change induced by paraquat in the lungs consisted of vacuolization and degeneration of the membranous pneumocytes (type I), followed by increase of collagen and reticulum in the basement membranes, proliferation of granular pneumocytes (type II). Swelling of the endothelial cells, proliferation of fibroblasts, and increase of the number of macrophages followed. A well developed lung lesion showed areas where the alveoli were completely obliterated or filled with an amorphous material which either formed a lattice reminiscent of surfactant or formed large whorls of a very electrondense material simliar to the material observed in the lamellar bodies. Fletcher and Wyatt (1970) recently reported a large increase in arachidonic acid and a small increase in cholesterol ester in the lungs of rats 6 days but not 2 days after poisoning with paraquat. The same authors found that the fatty acid composition of normal lung macrophages and peritoneal macrophages differed in many respects. It is possible that the increase in the fatty acids is due to the material accumulated in the alveoli illustrated in Fig. 4. Since this

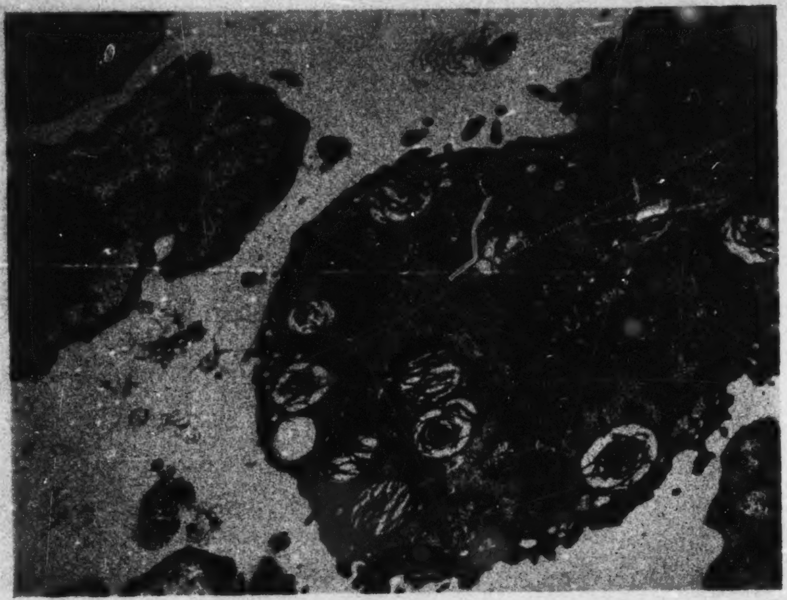

FIG. 6. Granular (type II) pneumocyte which contains light areas filled with a granular material. Lead citrate, uranyl acetate, ×9,864.

material does not accumulate in the early period of paraquat poisoning it would explain why an increase in the fatty acids were not found 2 days after paraquat poisoning. Kikkawa and Suzuki (1972) described a material in lung alveoli of rats that were injected with a hypocholesteremic drug AY 9944 (trans-1, 4-bis(2-chlorobenzylamino methyl) cyclohexane dihydrochloride). This material resembles morphologically the substance within the alveolar spaces described in this paper. Kikkawa and Suzuki also observed accumulation of electrondense granular and membranous bodies within the endothelial, the type I epithelial and the bronchiolar epithelial cells, findings not made in our paraquat treated rats. Kikkawa and Suzuki speculate that the accumulated material in the lungs resulted from an accelerated synthesis of surfactants or an abnormal clearance of the material. The membranous (type I) pneumocyte is instrumental in the removal of surfactant as Kikkawa and Suzuki point out. The membranous pneumocytes are the first cells affected by paraquat (Kimbrough and Gaines, 1970). They would subsequently not be able to remove any accumulated surfactant particularly not if it were abnormal. According to Sharp, Ottolenghi and Posner (1972) paraquat accumulates in lung and muscle, and work with unicellular cultures has confirmed that alveolar and peritoneal macrophages are much more readily killed by paraquat than are fibroblast (Corning, Fletcher and Swan, 1969) which may explain the overgrowth of fibroblasts in the lung. It would be interesting to establish whether the paraquat accumulated in the lung is primarily present in the lung tissue proper or in lung washings. It would also

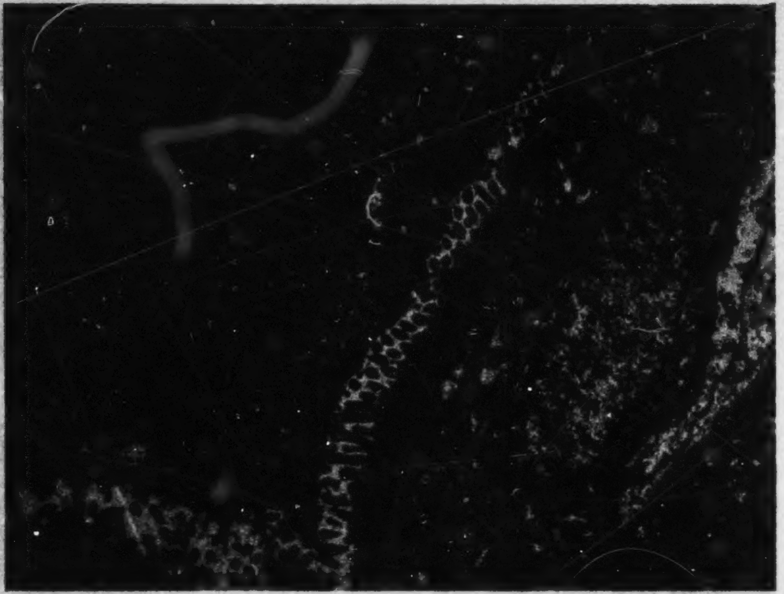

FIG. 7. A great deal of surfactant is seen. In contrast to the material observed in Figs. 3 and 4 this material is not closely attached to the cell wall. Lead citrate, uranyl acetate, ×9,864.

be worth while to examine whether expectorants would be useful in removing the electron-dense material observed in the alveoli.

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Mr. Richard Moore assisted with the animal experiments and Mrs. Annie Ruth Alford and Mrs. Linda Anderson prepared the tissue for light and electron microscopic study.

REFERENCES

1. ALMOG, C. H., AND TAL, E. (1971). Death from paraquat after subcutaneous injection. *Brit. Med. J.* 3, 721.
2. BROOK, R. E. (1971). Ultrastructure of lung lesions produced by ingested chemicals. I. Effect of the herbicide paraquat on mouse lung. *Lab. Invest.* **25**, 536–545.
3. CORNING, D. M., FLETCHER, K., AND SWAN, A. A. B. (1969). Paraquat and related bipyridyls. *Brit. Med. Bull.* **25**, 245–249.
4. FLETCHER, K., AND WYATT, J. (1970). The composition of lung lipids after poisoning with paraquat. *Brit. J. Exp. Pathol.* **51**(6), 604–610.
5. KIKKAWA, Y., AND SUZUKI, K. (1972). Alteration of cellular and acellular alveolar and bronchiolar walls produced by hypocholesteremic drug AY 9944. *Lab. Invest.* **26**, 441–447.
6. KIMBROUGH, R. D., AND GAINES, T. B. (1970). Toxicity of Paraquat to rats and its effect on rat lungs. *Tox. Appl. Pharmacol.* **17**, 679–690.
7. MODÉE, J., IVEMARK, B., AND ROBERTSON, B. (1972). Ultrastructure of the alveolar wall in experimental paraquat poisoning. *Acta Pathol. Microbiol. Scand. Sec. A* **80**, 54–60.

8. Nienhaus, H., and Ehrenfeld, M. (1971). Pathogenesis of lung disease in paraquat poisoning. *Beitrag. Pathol.* 142, 244–267.
9. Sharp, W. C., Ottolenghi, A., and Posner, H. S. (1972). Correlation of paraquat toxicity with tissue concentrations and weight loss of the rat. *Tox. Appl. Pharmacol.* **22**, 241–251.
10. Smith, P. (1971). A light and electron-microscopic study of the pulmonary lesions induced in rats by paraquat. Abst. 28, *J. Pathol.* 104, 7.
11. Toner, P. G., Vetters, J. M., Splij, W. G. S., and Harland, W. A. (1970). Fine structure of the lung lesion in a case of paraquat poisoning. *J. Pathol.* **102**(3), 182–185.
12. Vijeyaratnam, G. S., and Corrin, B. (1971). Experimental paraquat poisoning. A histological and electron-optical study of the changes in the lung. *J. Pathol.* **102**(2), 123–129.

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PUBLICATION

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Environmental Protection Agency

Brief Communication: Pancreatic-Type Tissue in Livers of Rats Fed Polychlorinated Biphenyls [1]

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SUMMARY—Pancreatic-type tissue in livers of Sherman strain rats is described. This lesion has not been reported previously. The pancreatic-type tissue was observed in livers of rats fed polychlorinated biphenyls (Aroclor 1254) for 6 months. The cells of the tissue had the same staining characteristics as salivary gland epithelium. The lesion was present in 15 of 36 livers examined.—J Natl Cancer Inst 51: 679–681, 1973.

DIETARY EXPOSURE of Sherman strain rats to polychlorinated biphenyls [500 parts per million (ppm) Aroclor 1254] for 6–8 months produces extensive areas of adenofibrosis in their livers. Liver enlargement, fatty metamorphosis, accumulation of brown pigment in hepatic macrophages, and Kupffer cells are also seen (*1, 2*).

Grossly adenofibrosis is firm and grayish white, with a pitted or granular surface. On microscopic examination it consists of fibrosis and proliferated, atypical-appearing glandular epithelium that forms ducts and cysts. The cysts usually contain mucus or cellular debris.

Edwards and White (*3*) reported a detailed study of adenofibrosis of the liver. Synonyms for adenofibrosis are cholangiofibrosis, bile-duct proliferation, bile-duct adenomatosis, and fibroadenoma. According to Edwards and White adenofibrosis can be produced in rats by *p*-dimethylaminoazobenzene. Bennett et al. (*4*) observed this when they fed rats a mixture of chlorinated naphthalenes and chlorinated biphenyls. After a critical review of the histopathogenesis of this lesion, Stewart and Snell concluded that it did not represent a precancerous change (*5*). In addition to the adenofibrosis observed, we also noticed small clusters of glandular cells within areas of adenofibrosis whose appearance differed strikingly from the epithelial cells normally in adenofibrosis. These clusters of glandular cells resemble pancreatic tissue in appearance and staining properties. The glandular cells resembling pancreatic tissue are described here.

Clusters of pancreatic-type cells were seen in 15 of 36 rat livers. These cell clusters, resembling the exocrine glandular cells of the pancreas, had a dark nucleus at the cell base and were columnar in shape. They had highly eosinophilic cytoplasm and formed acini (figs. 1, 2). They were most often found with foci of adenofibrosis in the livers, but were also in areas where the hepatic parenchyma was fairly normal, immediately adjacent to blood vessels in the center of the lobules. The pancreatic-type tissue, as these formations are probably best called, occurred usually in several different areas of the liver and in different areas of the same section. In adenofibrosis areas it was adjacent to the glandular component of that lesion (fig. 2), or surrounded by extensive fibrosis (fig. 1). Special stains for esterase (Animal Histology Procedures, NIH 1972), esterase with sodium taurocholate, and protein-bound tryptophan (*7*) were used. The stain for esterase with and without sodium taurocholate showed red cytoplasm; the stain for tryptophan gave the cytoplasm of these cells a blue granular appearance. These staining reactions suggest salivary gland tissue.

During the 10 years of work with rats of the Sherman strain, heterotopic pancreatic tissue has never been seen in the liver, although it has been reported in the stomach, duodenum, jejunum, Meckel's diverticulum (*8*), and gallbladder (*9*).

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[1] Received April 13, 1973; accepted April 25, 1973.

[2] I gratefully acknowledge the advice and help of Dr. K. C. Snell, Dr. H. L. Stewart, and Dr. M. K. Deringer, National Cancer Institute, Bethesda, Md.

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The cells described may originate from bile ducts; however, there is no evidence to substantiate this suggestion. Dr. H. L. Stewart and Dr. K. C. Snell kindly reviewed slides showing pancreatic-type tissue and did not recall ever having seen this type lesion.

REFERENCES

(*1*) KIMBROUGH RD, LINDER RE, GAINES TB: Morphological changes in liver of rats fed polychlorinated biphenyls. Arch Environ Health 25:354–364, 1972

(*2*) KIMBROUGH RD, LINDER RE, BURSE VW, et al: Adenofibrosis in the rat liver with persistence of polychlorinated biphenyls in adipose tissue. Submitted for publication

(*3*) EDWARDS JE, WHITE J: Pathologic changes with special reference to pigmentation and classification of hepatic tumors in rats fed *p*-dimethylaminoazobenzene (butter yellow). J Natl Cancer Inst 2:157–183, 1941

(*4*) BENNETT GA, DRINKER CK, WARREN MF: Morphological changes in the livers of rats resulting from exposure to certain chlorinated hydrocarbons. J Indust Hyg Tox 20:97–123, 1938

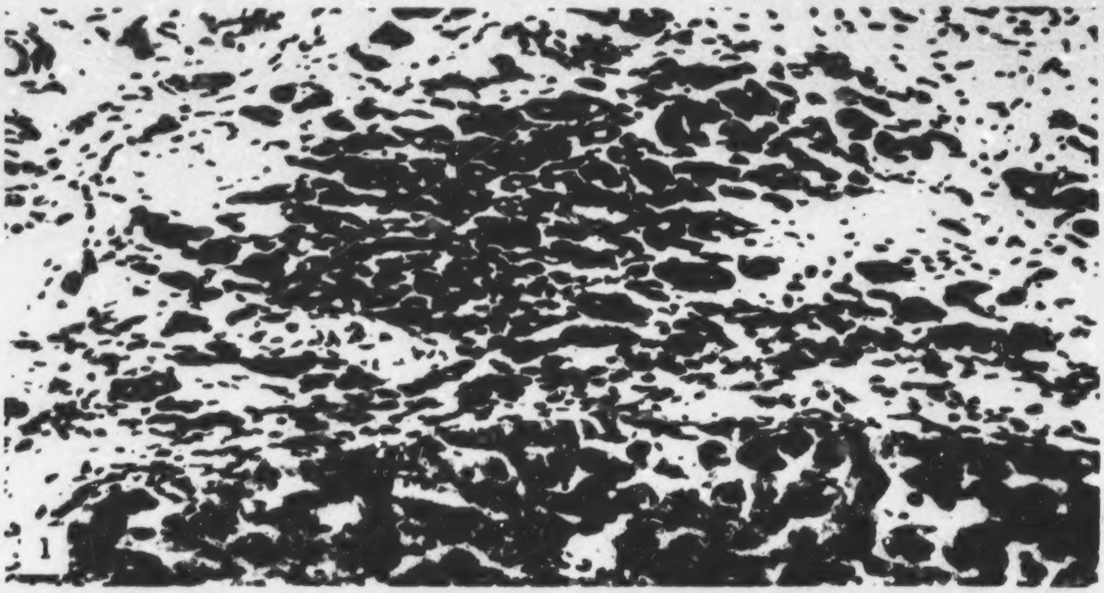
(*5*) STEWART HL, SNELL KC: The histopathology of experimental tumors of the liver of the rat. A critical review of the histopathogenesis. Acta Un Internat Contra Cancr 13:770–803, 1957

(*6*) SANDERS BJ: Animal Histology Procedures. DHEW Publication No. (NIH) 72–275

(*7*) GLENNER J: Hist Chem Cytochem 5:297, 1957

(*8*) ANDERSON WA: Pathology. St. Louis, The C.V. Mosby Company, 1961, p 874

(*9*) MANN FC: An accessory pancreas in the wall of the gallbladder of a dog. Anat Rec 23:351, 1922



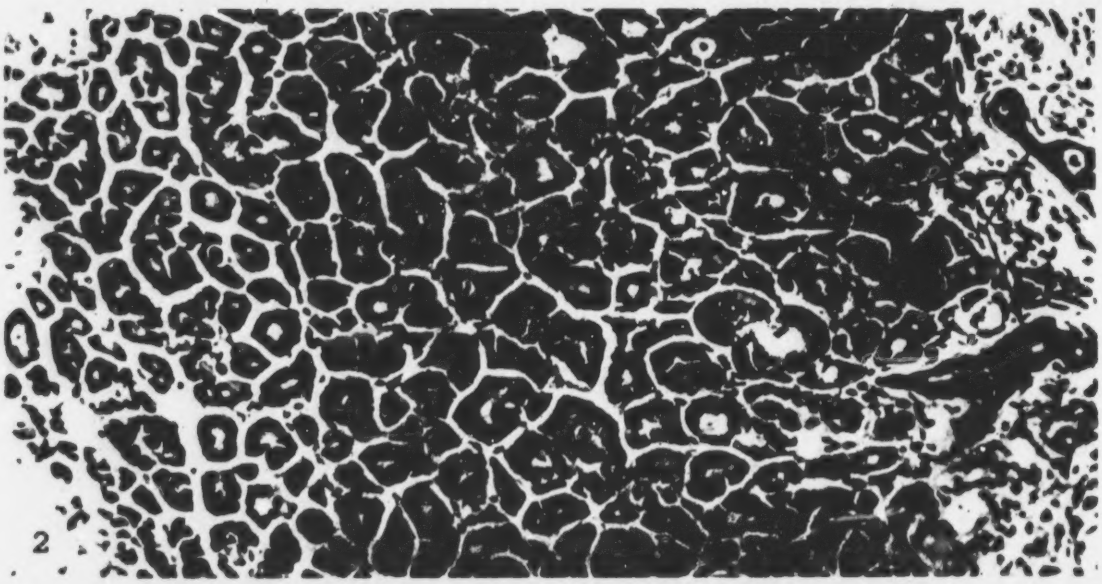


FIGURE 1.—Section of rat liver showing fibrous component of adenofibrosis are. *Note* cluster of small glandular cells forming acini (*center*). Cells show pancreatic-type tissue. Surrounding tissue shows primarily fibrous tissue. Hematoxylin and eosin. × 150

FIGURE 2.—Section of rat liver shows red staining reaction of cytoplasm of pancreatic-type tissue. Cell cluster in adenofibrosis area where epithelial component of adenofibrosis predominated. *Arrow* shows some of these glands. Esterase stain with taurocholate. × 500

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PEDIATRICS

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SUPPLEMENT

HEXACHLOROPHENE—Its Usage in the Nursery

Proceedings of a Conference held in Stowe, Vermont, under the auspices of the University of Vermont School of Medicine, June 1972

NOTE

Unfortunately we were not able to purchase individual reprints. The entire supplement in which this article appears can be purchased from the American Academy of Pediatrics, Evanston, Illinois 60204

Review of Recent Evidence of Toxic Effects of Hexachlorophene

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From the United States Environmental Protection Agency, Chamblee, Georgia

ABSTRACT. The published data on the toxicity of hexachlorophene in animals and man are discussed. Studies performed in the author's laboratory including hexachlorophene blood level determinations in animals and man are also reviewed. Hexachlorophene can produce paralysis in rats, rabbits, cats, and pigs and blindness in sheep. Microscopic examination of the brains of rats and monkeys given repeated doses of hexachlorophene shows status spongiosus of the white matter and normal gray matter. Small amounts of hexachlorophene are absorbed through the skin of animals as well as man. Aside from the neurological effects of hexachlorophene, the chemical uncouples oxidative phosphorylation which may cause body temperature elevation.

Pediatrics, 51:391, 1973, HEXACHLOROPHENE, TOXIC EFFECTS IN ANIMALS AND MAN.

THE literature on the toxicity of hexachlorophene was recently reviewed by Gump[1] and also by Kimbrough.[2]

Toxicity studies with hexachlorophene were performed in Sherman strain rats in our laboratory.[3] The acute oral LD_{50} for adult male rats was 66 mg/kg, for females 56 mg/kg, and for weanlings 120 mg/kg. In a reproduction study we found that the survival of offspring was slightly reduced in the F_1 generation when the parent rats were fed 100 ppm (8 mg/kg/day) hexachlorophene in their diet. They were mated 54 and 166 days after onset of exposure. The rats were fed the treated diets also during mating, gestation, and lactation. A dietary level of 20 ppm (1.5 mg/kg/day) did not affect reproduction.

A very pronounced effect on the brain was produced in rats fed hexachlorophene at the rate of 25 mg/kg/day. This effect could be observed microscopically in adult rats after they had been fed the hexachlorophene-containing diets for 4 days. A slight, but significant 6.5% increase in the wet weight of the brain over that of the controls was noticed after 2 weeks of exposure to diets containing 500 ppm (an average of 25 mg/kg/day) of hexachlorophene. An 11% increase in the wet weight of the brain over that of the controls was found after 4 weeks' exposure. When 500 ppm of hexachlorophene[4] were fed for 14 weeks, the wet weight of the brain averaged 2.60 gm, while the brains of the controls averaged 1.99 gm, which represents an increase in wet weight of 30.6%. The final body weights of these poisoned rats averaged 247 gm and those of the controls averaged 322 gm. Light microscopic examination of the brains of rats exposed to 500 ppm of hexachlorophene in the diet showed highly vacuolated white matter (status spongiosus) throughout the entire brain and spinal cord. The gray matter was normal. Electron microscopic examination of the white matter revealed that the vacuoles were due to a split in the interperiod line of the myelin sheath which had dilated and was probably filled with fluid. Occasionally, strands of myelin and granular material were observed within the vacuoles. At a dietary concentration of 100 ppm (5 mg/kg/day for 14 weeks) only a few rats showed vacuolated areas of the white matter. These areas were small and focal instead of diffuse. The other organs of these animals were normal grossly and microscopically. Four-week-old weanling rats showed status spongiosus of the white matter of the brain after a single dose of 100 mg/kg of hexachlorophene. The hexachlorophene was given by stomach tube in peanut oil. The brain lesion was most pronounced 4 days after dosing.

When a 3% concentration of hexachlorophene in propylene glycol or a detergent solution was applied daily to the skin of the back of rats for 30 days at the dosage levels of 48, 24, 12, and 0 mg/kg, erythema and ulceration of the skin developed at the dosage levels of 48 and 24 mg/kg. At the dosage level of 48 mg/kg the brains of all rats

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Presented at Stowehof Lodge, Vermont, June 15–16, 1972.

TABLE I

Hexachlorophene (HCP) Concentration
ppm in Blood
(ppm = μg HCP/g of whole blood)

	Infant		
	Cord *N = 50*	*Discharge* *N = 50*	*Adult General Population* *N = 14*
Range	0.003–0.100	0.009–0.646	<0.003–0.080
Mean	0.022	0.109	0.023
±S.E.	0.003	0.016	0.006

showed status spongiosus of the white matter. About half of the brains of the rats given 24 mg/kg/daily were affected. The brains of the rats with daily dermal exposure to 12 mg/kg were normal. The effect of hexachlorophene dissolved in propylene glycol was the same as when it was given in the detergent solution.

Monkeys have also shown this lesion. In a study conducted in another laboratory, newborn monkeys were washed daily with a 3% hexachlorophene solution in a detergent. They developed a diffuse status spongiosus of the white matter.[5] Santolucito[6] studied the effect of hexachlorophene in adult monkeys. He dosed squirrel monkeys each orally with 0, 1, 5, and 15 mg/kg/day for 28 days. The visual evoked potential was reduced in all exposed animals. The brains of these monkeys were essentially normal on microscopic examination. He then injected squirrel monkeys subcutaneously daily with 5 mg/kg of hexachlorophene for 37 days. The visual evoked potential in these animals was also reduced and a moderate diffuse status spongiosus of the white matter of the brain was observed. Brain tissue of the monkeys was also analyzed for hexachlorophene. Final hexachlorophene levels in the brain ranged from 0.18 ppm to 0.49 ppm. Monkeys that were given subcutaneous injections of 15 mg/kg/day for 1 week died. Their hexachlorophene brain levels were 0.8 ppm. The brains of the squirrel monkeys that died were not examined microscopically. (Recovery rate in brain was 75% in Santolucito's work).

Rats, rabbits,[7] pigs,[8] and cats (Dr. Stephen Krop, personal communication) show hindleg paralysis after exposure to large doses of hexachlorophene. In cats the pressure of the spinal fluid was increased. Sheep, according to Udall and Malone,[9] become blind. This blindness was irreversible while the paralysis in rats was reversible.[4] The brain lesion gradually resolves when rats are no longer exposed to hexachlorophene.[4] Other chemicals that produce status spongiosus of the white matter of the central nervous system in various species are triethyltin,[10] isoniazid,[11] and the halogenated benzanilide, 2-acetoxy-4-chloro-3,5-diiodobenzanilide.[12] Other halogenated salicylanilides may also cause this effect,[13] such as rafoxanide [3,5-diido-3'-chloro-4'-(p-chloro-phenoxy) salicylanilide].

The hexachlorophene levels in blood and various tissues of rabbits and rats that had been exposed to the chemical were also determined in our laboratory. We found in rabbits[14] that the peak hexachlorophene blood level was reached 7 hours following a single dermal application of 10 mg/kg hexachlorophene. Adult female rats that had been fed 100 ppm hexachlorophene in their diet for 258 days showed blood levels of 0.985–1.48 ppm with a mean of 1.21 ppm.[15] At this dietary level some rats demonstrated mild focal vacuolization of the white matter of the brain.

The blood levels of rats fed 500 ppm hexachlorophene in their diet for 55 days averaged about 8.5 ppm. When comparing blood levels it is important to know whether plasma or whole blood was analyzed for hexachlorophene. Flores and Buhler[16] found that in blood incubated with hexachlorophene 87% was bound to albumin in the blood of the newborn infant; 82% was bound to albumin in the blood of the rat. An additional significant amount was bound to the red blood cell membrane in both instances. Another important factor is the recovery rate. Our recovery rate for blood is now 95%. At the time we did the studies discussed below it was only 75%.

We determined hexachlorophene levels in 14 blood samples from the general population,[15] in 50 cord bloods and 50 blood samples from the same infants at the time of discharge from the hospital after birth.[17] Tables I and II give the results. The infants were washed daily with a 3% hexachlorophene-detergent solution. The number of washings and the interval after the last washing at the time the blood samples were obtained varied. The blood level determinations were done in collaboration with Drs. Nathenson and Finberg from the Albert Einstein College of Medicine.

These findings and those of Ulsamer, *et al.*[18] indicate that small amounts of hexachlorophene are absorbed through the intact human skin. This fact was also observed in rats by Carroll, *et al.*[19] who found that small amounts of radioactive hexachlorophene were absorbed through the intact skin of the tail of the rat.

When Curley and Hawk[15] studied the elimination of hexachlorophene from rats that had been fed 25 mg/kg/day for 55 days, they found that elimination is quite rapid at first. Half-life in kidney, liver, and blood is 10 hours. Half-life in the brain is about 19 hours. Excretion after a single dose follows the same pattern.

Caldwell, *et al.*[20] found hexachlorophene to be a very potent uncoupler of oxidative phosphorylation. Following a sublethal oral dose the maximal effect on oxidative phosphorylation occurred in the liver mitochondria about 12 to 15 hours after dosing and complete recovery required about 36 hours. Other well known uncouplers are pentachlorophenol and 2,4-dinitrophenols. Hexachlorophene was at least as or more effective. Cammer and Moore[21] also observed that hexachlorophene uncouples oxidative phosphorylation in brain and liver mitochondria. Hexachlorophene also inhibits cytochrome oxidase.[20,22] However, the inhibition of cytochrome oxidase seems to be of secondary importance. Strong binding of hexachlorophene to mitochondrial protein also occurs.[20]

A substantial increase in ATPase activity was evident in liver mitochondria. In rats receiving hexachlorophene orally (20 mg/kg) the ATPase activity of liver mitochondria isolated 18 hours later was three times that observed in control rats, but increasing the dose to 100 mg/kg did not result in a further ATPase increase.[20] Uncouplers of oxidative phosphorylation usually cause an elevation in body temperature. Pentachlorophenol is a good example of this. Nakaue, *et al.*[23] found that hexachlorophene elevated the body tem-

TABLE II

THE CONCENTRATION OF HEXACHLOROPHENE (ppm = μg/g) IN INFANT BLOOD AT BIRTH AND AT HOSPITAL DISCHARGE AFTER WASHINGS WITH HEXACHLOROPHENE SOLUTIONS

Infant Number	*Sex*	*Weight (gm)*	*Number of Washings*	*Concentration of Hexachlorophene*		
				Days in Hospital	*Cord Blood*	*Discharge Blood*
1	F	3,380	2	2	.010	.009
2	F	3,572	3*	2	.007	.010
3	F	3,884	3*	2	.007	.011
4	M	2,722	3	2	.011	.014
5	F	2,552	3	2	.008	.020
6	F	3,204	3	2	.008	.022
7	M	3,450	4	2	.005	.023
8	M	2,553	4	2	.009	.025
9	M	3,175	3	2	.007	.032
10	F	2,438	4	1	.010	.036
11	F	3,063	5	3	.011	.040
12	M	3,147	3*	2	.009	.044
13	M	2,863	3	2	.006	.045
14	F	2,948	3	2	.003	.046
15	F	3,110	5	2	.005	.046
16	M	2,466	3	2	.004	.048
17	M	3,175	3	2	.014	.049
18	M	3,175	3	3	.020	.052
19	M	2,637	3*	2	.011	.053
20	M	4,338	3	2	.100	.056
21	M	3,317	4*	3	.065	.065
22	F	2,381	5	2	.007	.070
23	M	3,345	5	3	.008	.071
24	M	2,438	3*	2	.010	.072
25	M	2,041	7	3	.031	.078
26	M	3,289	6*	7	.005	.080
27	F			2	.022	.081
28	M	3,317	3	2	.111	.081
29	F	2,750	2*	2	.006	.087
30	F	3,090	2	2	.017	.093
31	F	2,920	3*	2	.014	.095
32	F	3,402	4*	3	<.003†	.100
33	M	3,657	3	2	.182	.106
34	M	3,232	3	2	.086	.114
35	M	3,204	3	2	.010	.119
36	F	2,183	3	2	.021	.121
37	M	3,062	3	2	.027	.121
38	F	2,637	4	5	.004	.132
39	M	3,289	4	2	.013	.149
40	F	3,232	3	2	.016	.151
41	M	3,686	3*	3	.029	.162
42	M	3,450	5*	5	.040	.170
43	F	3,062	5	3	.010	.183
44	M	2,263	4*	4	.006	.202
45	M	2,293	9	8	.003	.234
46	M	2,070	14	11	.003	.250
47	F	3,062	4	3	.035	.275
48	M	3,033	4	6	.010	.326
49	M	4,083	5	9	.041	.352
50	M	3,097	5	2	.011	.646

* Probable number of washes.
† None detected.

perature in rats. In animals poisoned with an uncoupling agent rigor mortis sets in very rapidly after death and is very pronounced. This has been described for pentachlorophenol and we have also observed it in rats poisoned with hexachlorophene.

REFERENCES

1. Gump, W. S.: Toxicological properties of hexachlorophene. J. Soc. Cosmetic Chemists 20:173, 1969.
2. Kimbrough, R. D.: Review of the toxicity of hexachlorophene. Arch. Environ. Health, 23:119, 1971.
3. Gaines, T. B., and Kimbrough, R. D.: The oral and dermal toxicity of hexachlorophene in rats. Toxicol. Appl. Pharmacol., 19(2):375, 1971.
4. Kimbrough, R. D., and Gaines, T. B.: Hexachlorophene effects on the rat brain: Studies of high doses by light and electron microscopy. Arch. Environ. Health, 23:114, 1971.
5. FDA Bulletin. Hexachlorophene and Newborns. Dec. 1971.
6. Santolucito, J. A.: Electroencephalograms and visual evoked potential of the squirrel monkey fed hexachlorophene. Tox. Appl. Pharm., 22(2):276, 1972.
7. Balazs, T.: Measurement of acute toxicity. *In* Paget, G. E., Ed.: Methods in Toxicology. Philadelphia; F.A. Davis. Co., p. 60, 1970..
8. Larson, D. L.: Studies show hexachlorophene causes burn syndrome. Hospitals, J. Amer. Hosp. Ass., 42:63, 1968.
9. Udall, V., and Malone, J. C.: Optic nerve atrophy after drug treatment. *In:* The Problems of Species Difference and Statistics in Toxicology, Vol. XI. Amersterdam: Excerpta Medica Foundation, pp. 244-248, 1970.
10. Magee, P. N., Stoner, H. B., Barnes, J. M.: The experimental production of oedema in the central nervous system of the rat by triethyltin compounds. J. Path. Bact., 73:107, 1957.
11. Adornato, B., and Lampert, P.: Status spongiosus of nervous tissue. Acta Neuropath., 19:271, 1971.
12. Kurtz, S. M., Schardein, J. L., Fitzgerald, J. E., and Kaump, D. H.: Toxicologic studies with a halogenated salicylamide. Tox. Appl. Pharm., 14:81, 1969.
13. Brown, W. R., Rubin, L., Hite, M., and Zwickey, R. E.: Experimental papillodema in the dog induced by salicylamide. Tox. Appl. Pharm., 21:532, 1972.
14. Curley, A., Hawk, R. E., and Linder, R. E.: Hexachlorophene II. Percutaneous absorption in rabbits. Presented at the 161st National Meeting of the American Chemical Society, March 28-April 2, 1971, Los Angeles, California.
15. Curley, A., and Hawk, R. E.: Hexachlorophene I. Analysis in body fluids and tissues of experimental animals. Presented at the 161st National Meeting of the American Chemical Society, March 28-April 2, 1971, Los Angeles, California.
16. Flores, G., and Buhler, R.: In vitro hemolysis by chlorinated bisphenols. The role of hexachlorophene-protein interaction in the toxicity of chlorinated bisphenols. (Abst. 1725) Fed. Proc., 31:520, 1972.
17. Curley, A., Hawk, R. E., Kimbrough, R. D., Nathenson, G., and Finberg, L.: Dermal absorption of hexachlorophene in infants. Lancet, 2:296, 1971.
18. Ulsamer, A. G., Yoder, P. D., and Marzulli, F. N.: Determinations of hexachlorophene in human and experimental animal tissues. Tox. Appl. Pharm., 22(2):276, 1972.
19. Carroll, F. E., Salak, W. W., Howard, J. M., and Pairent, F. W.: Absorption of antimicrobial agents across experimental wounds. Surg. Gynec. Obstet., 125:974, 1967.
20. Caldwell, R. S., Nakaue, H. S., and Buhler, D. R.: Biochemical lesion in rat liver mitochondria induced by hexachlorophene [2,2'methylene bis (3,4,6-trichlorophenol)] Biochem. Pharm., in press.
21. Cammer, W., and Moore, C. L.: The effect of hexachlorophene on the respiration of brain and liver mitochondria. Biochem. Biophys. Res. Communications, 46:1887, 1972.
22. Gould, B. S., Frigerio, N. A., and Lebowitz, W. B.: The action of the bisphenolic compounds on succinoxidase, cytochrome c oxidase and lactic dehydrogenase of animal tissue. Archs. Biochem. Biophys., 56:476, 1955.
23. Nakaue, H. S., Dost, F. N., and Buhler, D. R.: Studies on the toxicity of hexachlorophene in the rat. In preparation.

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Toxic Effects of the Herbicide Paraquat*

Renate D. Kimbrough, M.D.

Paraquat is a general weed killer of the bipyridyl family of herbicides (1, 1'-dimethyl 4,4' bipyridylium). Paraquat is available either as the dichloride or dimethyl sulfate salt, both compounds are water soluble. In the United States the following formulations are available: Orthoparaquat, a 29.1 percent solution, Ortho dualparaquat, a 42 percent solution, and Ortho Spot, weed and grass killer, a 0.44 percent solution. Paraquat was developed in the 1950's, and came onto the market in Great Britain in 1962. It was registered for use in the United States in 1964. Registration also permits aerial spraying of diluted formulations.

According to a recent editorial in *Lancet*,[1] the total number of human deaths known to the manufacturer from paraquat poisoning is now 124 worldwide. In addition, 60 persons who have recovered from paraquat poisoning are also known. The fatal dose in adults is thought to be about 15 ml of a 20 percent solution, or a "mouthful" as it is described in most case histories. Formulations which contain 5 percent of paraquat if accidentally swallowed are usually not fatal. Fatal poisoning in almost all cases has been due to ingestion of the material. The initial symptoms of poisoning consist of burning of the mouth and throat, followed by nausea and vomiting. After a latent period of up to several days, increasing respiratory distress develops, a transient effect is also observed on kidney, liver and heart function and transient neurologic signs are seen, but death is usually the result of a progressive fibrosis and epithelial proliferation that occurs in the lungs.

Dermal exposure to the paraquat concentrate may result in severe skin irritation. Diluted spray solutions may produce slight to moderate irritation. Paraquat is absorbed through the skin. In toxicity studies conducted in our laboratory we found that the acute dermal LD_{50} in rats was 80 mg paraquat/kg body weight in males and 90 mg/kg in females, while the acute oral LD_{50} in male rats was 100 mg/kg and in females 110 mg/kg.[2] These findings indicate that there does not seem to be a great difference between the oral and dermal toxicity in rats.

McDonagh and Martin[3] reported four cases of paraquat poisoning in children. One of the patients, a three-year-old boy, was admitted to the hospital with superficial burns on his right thigh and hands 24 hours after he had spilled a 20 percent paraquat concentrate (Gramoxone) on his clothes. He had also been seen playing near a pool of the liquid. About six to eight hours after admission an erythematous rash appeared on his thigh and hands. The rash developed into a large first degree burn. No lesions were seen in his mouth. In spite of the fact that the child did not appear to have ingested any paraquat, it was present in his urine. This case illustrates that paraquat is also absorbed through human skin.

As pointed out earlier, paraquat is registered in certain instances for spraying by airplane in the United States. Fortunately, the particle size is large enough so that direct inhalation does not seem to present a problem unless high pressure spray or air blast equipment is used. According to Gage[4] the "no-effect" level for a "respirable" paraquat aerosol in the rat is about 0.1 mg/M^3 for repeated six-hour exposures, which would suggest that 0.1 mg/M^3 would not be excessive for occupational exposure. The threshold limit value for paraquat in workroom air in the United States set by the American Conference of Government Industrial Hygienists is 0.5 mg/M^3 at present.

Patients have on occasion had nose bleeds due to a local effect of paraquat on the nasal mucosa. If paraquat is splashed into the eye, particularly the paraquat concentrate, severe eye injuries have occurred,[5] and in

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*From the Environmental Protection Agency, Chamblee, Ga.

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the authors

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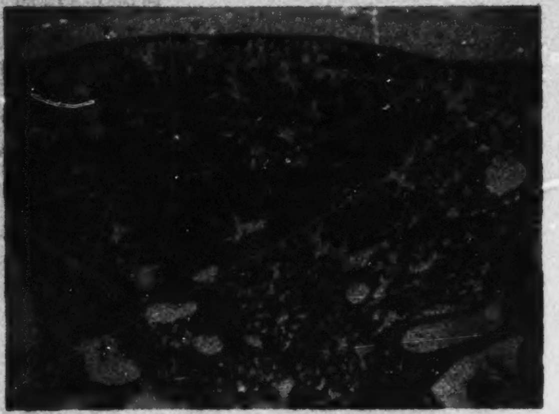

Figure 1. Section of lung of rat given 160 mg/kg paraquat by stomach tube. Rat died six days later. Alveoli contain edema fluid, inflammatory cells and macrophages (PAS stain, × 50).

addition, injuries to the fingernails, nail beds and skin have also been reported.[6] All of these injuries are primarily due to the local corrosive effect of this product.

Death following paraquat poisoning is usually the result of progressive fibrosis and epithial proliferation in the lungs. This effect has been described in human beings, rats, guinea pigs, mice and dogs.[1]

We[2] have studied the effect of paraquat on the lung of the Sherman rat. The early effect of paraquat after a single oral dose of 150 mg/kg by stomach tube, was pulmonary edema within 48 hours.

Examination of the affected lungs with the electronmicroscope showed swelling and large vacuoles in the cytoplasm of the membranous pneumocytes. Dark prominent granules were also seen in the cytoplasm and were thought to represent ribosomes. Widening of the basement membrane with separation of the epithelial cells from the basement membrane was noted. Pulmonary edema and red blood cells were observed in the alveoli.[2] A rat which died four days after a single oral dose of 160 mg/kg of paraquat in water showed firm dark lungs on gross inspection, and microscopic examination revealed edema and hemorrhage.

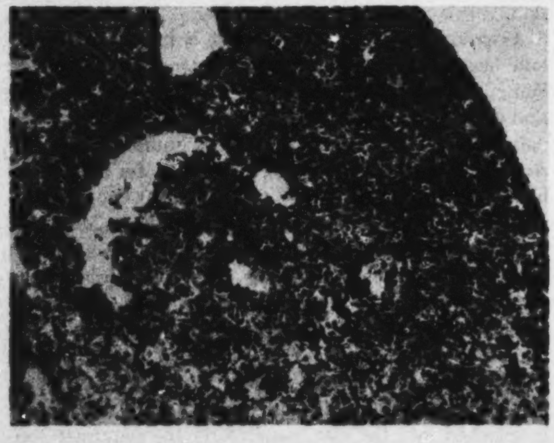

Figure 2. Section of lung of rat given 150 mg/kg of paraquat by stomach tube. Rat died ten days later. Note extensive areas of proliferation of fibrous and epithelial cells. Nuclei of some epithelial cells are enlarged hyperchromatic H&E (× 125).

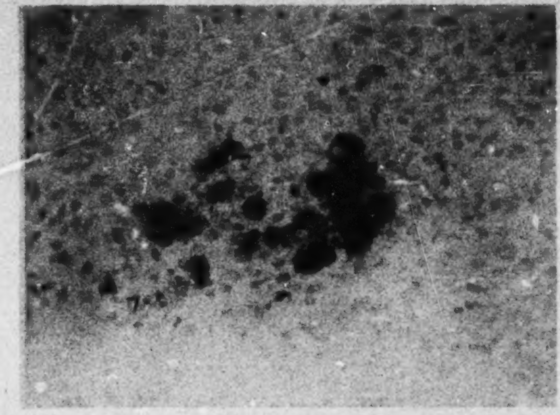

Figure 3. Section of lung from rat receiving 40 mg/kg paraquat, killed eight weeks later. *Note* pigment staining with Perl stain within macrophages (Perl stain × 500).

Rats dying six days after a single dose of paraquat showed an inflammatory reaction in the lungs (Fig 1). Polymorphonuclear leukocytes, macrophages and edema fluid were observed in the alveoli. Evidence of the earlier hemorrhage was still present. A brown pigment which stained positive for iron with Perl's stain was observed, and probably represented hemosiderin. A highly eosinophilic PAS-positive membrane lining the alveoli was also noted. In rats surviving ten days following a single toxic dose of paraquat the lungs showed beginning proliferation of fibrous and alveolar cells (Fig 2). Rats given a single oral dose of 40 mg/kg body weight, killed eight weeks later showed fairly extensive areas of consolidation. Microscopic examination of these firm grayish areas revealed extensive fibrosis and a brown pigment positive for iron with Perl's stain (Fig 3), which was consistent with hemosiderin findings. Slight proliferation of epithelial cells was also observed.

When rats were fed 500 ppm paraquat daily in their diet (20-30 mg/kg body weight) an occasional rat developed areas of consolidation.[3] These areas of consolidation contained proliferated epithelial cells which were surrounded by inflammatory cells and fibroblasts. A prominent amorphous material was also observed in the

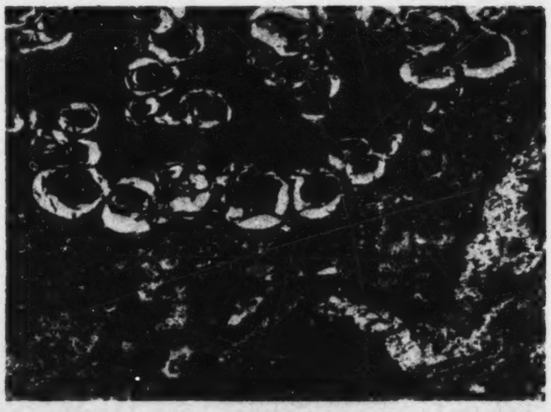

Figure 4. Electronmicrograph showing electron dense material in alveoli *(arrow)*. Lead citrate and uranyl acetate (× 6,745).

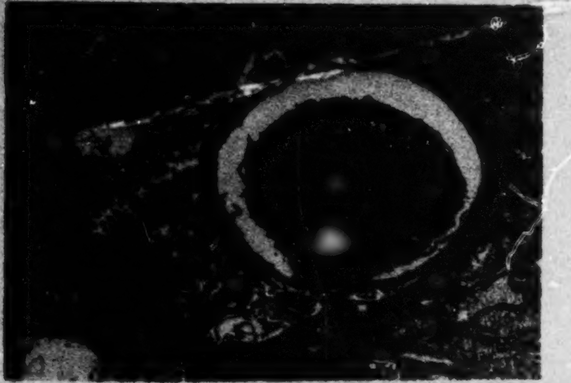

FIGURE 5. *Electronmicrograph* illustrating large "whorl" of electron dense material within cytoplasm of alveolar epithelial cell. Lead citrate, uranyl acetate (× 3,230).

alveoli. Electronmicroscopic examination (Fig 4,5) showed this material to be striated or to form whorls of electron dense material reminiscent of surfactant and lamellar bodies. Fletcher and Wyatt[9] recently reported a large increase in arachidonic acid and a small increase in the cholesterol esters in the lungs of rats six days after poisoning with paraquat. It is possible that the increase in the fatty acids is due to the material accumulated in the alveoli, which was just illustrated. A hypocholesteremic drug AY9944 apparently shows a similar accumulation of material within the alveoli of the lungs.[10]

There is a possibility that paraquat is absorbed by the lungs, then may become reactivated in the lung by oxygen. The treatment of patients suffering from paraquat poisoning with oxygen probably aggravates the lung lesion, and oxygen should therefore not be administered. Paraquat is absorbed on bentonite, fuller's earth and related materials, and becomes deactivated. These various absorbents are most effective when given orally within one hour or less after the poisoning by ingestion has occurred. Brown[11] has recommended the suspension of 300 gm of finely divided fuller's earth, previously sterilized to kill bacterial spores, in 1 liter of water to be given promptly after the initial lavage. Forced diuresis may also have an effect in treating the poisoning, although this has not been definitely proved. Paraquat is fortunately not well absorbed by the skin or the gastrointestinal tract. Therefore, most patients who have taken the 5 percent solution have survived. If about .05 mg/kg body weight paraquat in water is instilled directly into the trachea in rats, localized fibrosis is produced in the rat lung. This finding illustrates that not very much paraquat needs to reach the lung in order to produce fibrosis.[3]

In summarizing the sequence of events following a lesion on the lung caused by paraquat, the following seems to take place: the alveolar epithelium becomes vacuolated and degenerated; the alveoli fill with edema fluid; basement membranes are widened; and red blood cells appear in the alveoli. These events are followed by infiltration of the lung with inflammatory cells, fibrosis and epithelial proliferation, and accumulation in the alveoli of electron dense material that resembles lamellar bodies and surfactant.

REFERENCES

1 Anonymous: Paraquat poisoning. Lancet 11:1018-1019, 1971

2 Kimbrough RD, Gaines TB: Toxicity of paraquat to rats and its effect on rat lungs. Toxicol Appl Pharmacol 17: 679-690, 1970

3 McDonagh BJ, Martin J: Paraquat poisoning in children. Arch Dis child 45:425-427, 1970

4 Gage JC: Toxicity of paraquat and diquat aerosols generated by a size-selective cyclone. Effect of particle size distribution. Br J Ind Med 25:304-314, 1968

5 Cant JS, Lewis DRH: Ocular damage due to paraquat and diquat. Br Med J 2:224, 1968

6 Samman PD, Johnson ENM: Nail damage associated with handling of paraquat and diquat. Br Med J 1:818-819, 1969

7 Clark DG, McElligot TF, Hurst EW: The toxicity of paraquat. Br J Ind Med 23:126, 1966

8 Kimbrough RD, Linder RE: The ultrastructure of the paraquat lung lesion in the rat. (Environ Res in press)

9 Fletcher K, Wyatt J: The composition of lung lipids after poisoning with paraquat. Br J Exp Pathol 51:604-610, 1970

10 Kikkawa Y, Suzuki K: Alteration of cellular and acellular alveolar and bronchiolar walls produced by hypocholesteremic drugs AY9944. Lab Invest 26:441-447, 1972

11 Browne TD: Treatment of paraquat ingestion (letter to editor). Br Med J 3:580, 1971

DISCUSSION

Dr. Corrin: I think it is important to re-emphasize the point that you made, and that is the route by which paraquat reaches the lung. I know of no fatal cases of paraquat poisoning in which the route to the lung has been the respiratory tract. All the fatal cases that have been reported are those in which the paraquat is ingested. In our experimental preparations, the data agree closely with yours in that the epithelium is the major site of damage. There is some endothelial degeneration but the epithelial damage can proceed to complete necrosis with the endothelium being apparently quite normal. This is particularly interesting when one realizes that this toxic substance had reached the alveolar wall via the circulation and not via inhalation. This material is so extremely toxic that one might ask what we can do to reduce the hazard of this material. One might initially suggest that we ban the sale of this material but our farming friends tell us that this is an extraordinarily valuable chemical and a ban would put a great burden on them.

Dr. Kimbrough: I think it would be far better if the law could be changed and the dilute form of paraquat would be the only form available on the market. This would decrease the number of cases of such fatal poisonings.

Dr. K. Fisher: I would like to extend Dr. Kimbrough's remarks on the role of oxygen in paraquat poisoning. We have found that paraquat is far more rapidly and uniformly fatal in animals breathing an atmosphere of oxygen than in those breathing room air. We have also found that concentrations of paraquat which are bacteriostatic to aerobically grown *E coli* have no effect on anaerobic growth of *E coli*.

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Storage and Excretion of DDT in Starved Rats

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It is well known that some poisons, such as lead when stored in sufficient amount, may be mobilized from storage with the result that clinical illness appears or recurs. This phenomenon was first demonstrated experimentally for DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] by Fitzhugh and Nelson (1947). They showed that rats that had been fed diets containing DDT at concentrations of 600 parts per million (ppm) or more showed marked tremors when they were deprived of food completely. Rats deprived of all food after being fed 200 or 400 ppm showed increased irritability but no tremors. It has been assumed that these clinical effects resulted from a mobilization of DDT concurrent with the mobilization of body fat, but the effect of starvation on the concentration of DDT in various tissues and excreta has not previously been measured.

This paper reports the results of such measurements in rats that were starved and in suitable controls. The results confirm that DDT is, in fact, mobilized into the circulation and into the brain during starvation and thus explain the clinical effects.

METHODS

The rats used were 22- to 28-day-old weanlings of the Sherman strain. Their ordinary food was Purina Laboratory Chow.[1] They were maintained on this Chow in a ground form throughout the experiment, either with no added DDT or with recrystallized p,p′-isomer added at a concentration of 200 ppm. A total of 30 rats of each sex on added DDT and 24 rats of each sex on plain Chow (108 rats in all) were placed in large rabbit cages, 12 or fewer rats to the cage. The rats were kept in these cages until the 83rd day of the experiment. At this time, 24 rats of each sex and each dosage were put in separate, numbered cages and assigned, by means of a table of random numbers, to 3 groups of 8 rats each to receive the following treatment: group 1: to be sacrificed after 90 days of feeding ad libitum; group 2 to be sacrificed after 90 days of feeding ad libitum followed by 10 days of partial starvation; and group 3: to be sacrificed after 90 days of feeding ad libitum, 10 days of partial starvation, and 40 additional days of recovery on ad libitum feeding (total of 140 days).

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[1] The use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service.

The 6 rats of each sex (on 200 ppm of DDT) remaining in the large rabbit cages were saved and continued on their same dietary concentration of DDT until the end of the experiment at 140 days. The design of the experiment as given above, is summarized in Table 1.

TABLE 1

DOSAGE AND SACRIFICE SCHEDULE OF RATS

Group designation		Added DDT in diet (ppm)	Sacrificed
Males	Females		
1M	1F	200	After 90 days on DDT
2M	2F	200	After 100 days on DDT, including last 10 days on starvation
3M	3F	200	After 140 days on DDT, including 10-day starvation followed by 40-day recovery
4M	4F	200	After 140 days on DDT, no starvation
5M	5F	0	Same as group 1, except no DDT
6M	6F	0	Same as group 2, except no DDT
7M	7F	0	Same as group 3, except no DDT

Each rat remained on the same dietary concentration of DDT throughout the experiment; only the amount of food offered was varied intentionally in certain groups. The small differences in caging dictated by necessity or convenience were considered unimportant.

After separation into groups, animals in groups 1M, 1F, 5M, 5F, 6M, 6F, 7M, and 7F (Table 1) were individually caged in AMS-type cages, while those in groups 2M, 2F, 3M, and 3F were caged in separate compartments of large metabolism cages. Each metabolism cage was divided into four compartments so that the food consumption for each rat could be measured separately, while the feces and urine collections could be combined. Food consumption of all rats except those in groups 4M and 4F was measured daily for 7 days previous to starvation and, based on the values obtained, the rats to be starved were fed half the amount of the average normal daily intake for the group during the 10-day starvation period. The measured food intake of rats that were starved and then permitted to recover, is shown in Fig. 1A. Rats scheduled for sacrifice finished eating their food about 8 hours previous to time of sacrifice.

All rats were weighed at the beginning of the experiment, at intervals during the study, and at the time of death or sacrifice. The rats that were placed on starvation diets were weighed daily during the critical part of the experiment.

Separate daily collections of urine and separate daily collections of feces were made for groups 2M, 2F, 3M, and 3F and pooled by groups. The urine samples were stored in a deep freeze until time of analysis. Each collection of feces was dried for 24 hours in an oven at 70°C before storage in a deep freeze until time of analysis.

Blood, brain, liver, kidney, and fat tissues were taken from each rat at the time of sacrifice given in the schedule presented in Table 1.

At the time of sacrifice, as much blood as possible was collected via cardiac puncture under ether anesthesia. Heparin was used in the syringe to prevent clotting. The plasma was immediately extracted by the method of Bloor (1914). The plasma extracts were evaporated to dryness in a 75° C water bath with the aid of a gentle stream of air. DDT and DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene] in the residue were measured by the Schechter-Haller method as modified by Schechter *et al.* (1945).

Brain, liver, kidney, and fat tissues were weighed and placed in small screw-cap bottles and stored in a deep freeze until time of analysis. These tissues were analyzed for DDT and DDE by the Schechter-Haller method as modified by Mattson *et al.* (1953).

Urine samples were analyzed for DDA [2,2-bis(*p*-chlorophenyl)acetic acid], which is the principal metabolite of DDT excreted in the urine, using the method as modified by Schechter *et al.* (1945). Only trace amounts of other metabolites have been reported (Ofner and Calvery, 1945). All of these urinary metabolites of DDT that respond to the Schechter-Haller method give a red color, therefore all calculations were made on the basis of DDA. DDT, which responds to this method to give a blue color, was not found in the urine of these animals.

The dried, frozen samples of feces were ground in a Wiley mill until they would pass a 40-mesh screen. The dried and ground feces were nitrated directly using a severe nitrating condition (Jensen *et al.*, 1957) that gives more complete recovery of DDT and its metabolites in feces than does the regular method of Schechter and Haller. It has been shown that the metabolic products of DDT in the feces that respond to the Schechter-Haller method to give a red color are primarily DDA-like materials with only about 2% appearing as DDE (Jensen *et al.*, 1957). All Schechter-Haller-positive red-colored materials in the feces were, therefore, calculated as DDA.

RESULTS

Clinical

Reduction of food intake to half the normal amount caused the expected rapid reduction of body weight, as shown in Fig. 1. Rats of both sexes that had been fed DDT weighed slightly less than their controls before starvation. The amount of weight loss for males was greater than that for females, but the rate of loss was essentially the same for both sexes irrespective of DDT intake. In a similar way, the males and females regained during recovery the same percentage of original weight per unit time. The form of the curves for body weight during the recovery phase was explained by the food intake. Both food intake and body weight approached a steady state (involving slow increase of body weight) about the 120th day of the experiment.

Two rats of each sex that exhibited typical DDT tremor died during the starvation period. Analytical results from these animals were not used in preparation of Tables 2 through 7. The males showed tremor beginning on the third and fourth days and died on the fourth and tenth days, respectively, after the beginning of forced starvation. However, the first male to die failed to eat at all during the last 9 days before death. The females were observed in tremor 4 days before the beginning of starvation and died 4 and 10 days later, respectively. The first female to die ate a total of only 5.4 g of food during the last 8 days before death.

There were no signs of illness in any animal reported in Tables 2 through 7 except that 4 of the females in group 2F and 5 of those in group 3F fed DDT at a rate of 200 ppm showed hyperexcitability and slight to moderate tremors beginning on the seventh day of half rations. Those not sacrificed at the end of the starvation period quit having tremors 2 days after the return to ad libitum feeding. This clinical recovery oc-

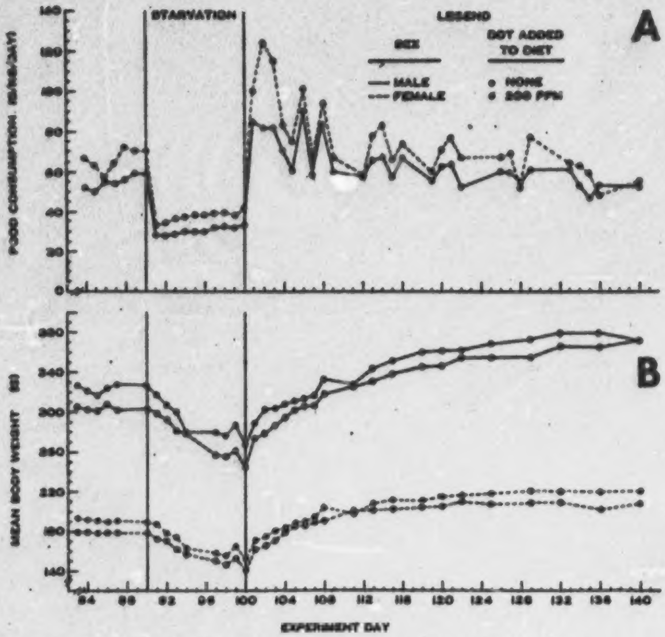

Fig. 1. Food intake (A) and body weight (B) of rats fed DDT.

curred even though the animals were eating more food (and thus more DDT) than at any other time during the experiment (Fig. 1A).

Storage

The concentrations of DDT and DDE found in different tissues are shown in Tables 2 through 7. Analysis of all tissues was made on the basis of total lipid weight and also fresh wet weights. The discussion that follows has been limited to values based on total extractable lipid weight.

It may be seen that partial starvation and accompanying weight loss led to an increased concentration of DDT and DDE in the fat of rats fed DDT at a concentration of 200 ppm (Table 2). The factor of increase

TABLE 2

DDT and DDE in the Fat of Rats Fed 200 ppm DDT in Their Diet

Group number	History	Statistical evaluation	DDT (ppm)				DDE (ppm)			
			Lipids		Tissue		Lipids		Tissue	
			Male	Female	Male	Female	Male	Female	Male	Female
1	90 Days on DDT prestarvation	Range	433–864	892–2729	373–735	729–2206	40–90	78–509	36–75	64–412
		Mean ± S.E.	621 ± 60	1447 ± 248	525 ± 50	1223 ± 205	51 ± 5	191 ± 53	51 ± 5	161 ± 44
		N	7	8	7	8	7	8	7	8
2	100 Days on DDT including last 10 days on starvation	Range	512–1511	1857–10204	258–1010	1477–2731	87–385	230–2199	68–257	183–535
		Mean ± S.E.	988 ± 116	4239 ± 1068	669 ± 91	2160 ± 151	216 ± 39	783 ± 255	142 ± 26	370 ± 45
		N	8	8	8	8	8	8	8	8
3	140 Days on DDT including 10 days' starvation followed by 40 days' recovery	Range	445–963	926–1553	384–826	739–1345	33–132	160–322	28–114	128–264
		Mean ± S.E.	624 ± 7	1176 ± 85	525 ± 67	987 ± 81	63 ± 15	241 ± 24	53 ± 13	203 ± 20
		N	6	6	6	6	6	6	6	6
4	140 Days on DDT, no starvation	Range	559–657	1013–1982	472–562	847–1746	40–63	96–280	33–53	80–224
		Mean ± S.E.	598 ± 12	1638 ± 150	506 ± 16	1418 ± 147	50 ± 3	195 ± 26	42 ± 2	168 ± 21
		N	6	6	6	6	6	6	6	6

for males (groups 1M and 2M) was 1.5 for DDT and 4.2 for DDE. The corresponding values for females (groups 1F and 2F) were 2.9 for DDT and 4.1 for DDE. The difference was significant ($P = < 0.05$) in each instance. Rats that were starved in the same way and then allowed to recover for 40 days (group 3) showed concentrations of DDT and DDE in the fat statistically indistinguishable from rats that had not undergone starvation (groups 1M and 1F and 4M and 4F) with the single exception that the females that had starved and recovered (group 3F) showed a statistically *lower* concentration of DDT ($P = < 0.05$) than unstarved females. These results indicate that the rats in groups 1M and 1F had reached an equilibrium of storage so far as fat was concerned sometime during the 90 days of feeding they received.

During the steady state, the storage of DDT and DDE in the fat was greater in female rats (groups 1F and 4F) than in males (groups 1M and 4M) and the difference in storage (2.3 to 2.7) exceeded the difference in dosage (1.15) resulting from the greater food intake of the females. The factors given here are similar to those based on other data, i.e., 1.5 to 3.8 for difference in storage and 1.25 for difference in food intake as reported earlier (Hayes, 1959).

In general, as shown in Table 3, the same results regarding the storage of DDT and DDE in fat were obtained for rats fed a diet with no intentionally added DDT but containing residues to the extent of less than 0.5 ppm. An exception was the failure of males to show any increase in the concentration of DDE in the fat immediately after starvation. This apparent exception may reflect only the difficulty of measuring very low concentrations of DDE. No attempt was made to measure the concentration of DDT or DDE in the plasma, viscera, or excreta of rats fed no added DDT because the amounts present were so small that they could not be determined with accuracy.

During the steady state of storage, there was no difference between the storage of DDT or DDE in males and females at this low dosage.

In rats fed DDT at a concentration of 200 ppm, the concentration of DDT and DDE in the plasma increased during limited starvation and returned to prestarvation levels after resumption of feeding (Table 4). The results are entirely compatible with the view that the concentration of DDT in the plasma was in continual equilibrium with that in the fat of the same animal, and that the same was true of DDE also.

The results for the storage of DDT and DDE in the brain (Table 5),

TABLE 3

DDT AND DDE IN THE FAT OF RATS FED < 0.5 PPM DDT IN THEIR DIET

Group number	History	Statistical evaluation	DDT (ppm)				DDE (ppm)			
			Lipids		Tissue		Lipids		Tissue	
			Male	Female	Male	Female	Male	Female	Male	Female
5	90 Days on DDT, prestarvation	Range	4–12	3–13	4–8	3–11	2–5	0.2–4	2–4	0.2–3
		Mean ± S.E.	8 ± 0.88	8 ± 2.0	6 ± 0.68	6 ± 1.0	3 ± 0.31	2 ± 0.57	3 ± 0.85	1 ± 0.45
		N	8	7	7	7	8	7	7	7
6	100 Days on DDT, including last 10 days on starvation	Range	8–30	9–88	8–22	7–30	0.9–10	1–44	0.8–7	1–12
		Mean ± S.E.	14 ± 3	42 ± 11	11 ± 2	19 ± 3	3 ± 1	17 ± 6	2 ± 0.85	7 ± 2
		N	7	7	7	7	7	7	7	7
7	140 Days on DDT including 10 days' starvation followed by 40 days' recovery	Range	6–11	4–11	5–10	4–10	2–5	2–5	2–4	1–4
		Mean ± S.E.	8 ± 0.51	8 ± 0.69	7 ± 0.56	7 ± 0.58	3 ± 0.31	3 ± 0.33	3 ± 0.30	3 ± 0.27
		N	8	8	7	8	8	8	7	8

TABLE 4

DDT AND DDE IN PLASMA OF RATS FED DDT AT A DIETARY LEVEL OF 200 PPM

Group number	History	Statistical evaluation	DDT (ppm)				DDE (ppm)			
			Lipids		Whole plasma		Lipids		Whole plasma	
			Male	Female	Male	Female	Male	Female	Male	Female
1	90 Days on DDT, prestarvation	Range	58–121	44–171	1–2	0.7–3.9	el[a]–170	el–161	el–2.85	el–2.71
		Mean ± S.E.	77 ± 6	120 ± 24	1.3 ± 0.11	2.0 ± 0.37	72 ± 24	88 ± 17	1.21 ± 0.40	1.48 ± 0.29
		N	8	8	8	8	8	8	8	8
2	100 Days on DDT including last 10 days on starvation	Range	50–154	84–282	0.8–2.6	1.4–4.7	58–170	85–306	1.0–2.9	1.4–5.1
		Mean ± S.E.	96 ± 14	192 ± 24	1.61 ± .24	3.2 ± 0.4	103 ± 13	177 ± 27	1.7 ± 0.22	3.0 ± 0.45
		N	7	8	7	8	7	8	7	8
3	140 Days on DDT including 10 days' starvation followed by 40 days' recovery	Range	65–99	97–182	1.1–1.7	1.6–3.1	51–82	56–140	0.9–1.4	0.9–2.4
		Mean ± S.E.	81 ± [illegible]	132 ± 13	1.4 ± 0.08	2.2 ± 0.23	72 ± 4	84 ± 12	1.2 ± 0.07	1.4 ± 0.20
		N	6	6	6	6	6	6	6	6
4	140 Days on DDT, no starvation	Range	48–96	17–250	0.81–1.62	0.28–4.2	28–61	41–108	0.48–1.05	0.68–1.81
		Mean ± S.E.	69 ± 7	107 ± 40	1.17 ± 0.11	1.8 ± 0.67	52 ± 5	74 ± 11	0.87 ± 0.08	1.3 ± 0.18
		N	6	5	6	5	6	5	6	5

[a] Below the experimental limits of the method.

TABLE 5

DDT AND DDE IN THE BRAIN OF RATS FED 200 PPM DDT IN THEIR DIET

Group number	History	Statistical evaluation	DDT (ppm)				DDE (ppm)			
			Lipids		Tissue		Lipids		Tissue	
			Male	Female	Male	Female	Male	Female	Male	Female
1	90 Days on DDT, prestarvation	Range	74–106	110–280	6–8	8–21				
		Mean ± S.E.	92 ± 4	162 ± 25	7 ± 0.19	12 ± 2	el*	el	el	el
		N	8	7	8	7	8	7	8	7
2	100 Days on DDT including last 10 days on starvation	Range	99–236	124–523	7–18	9–39	31–102		2–8	
		Mean ± S.E.	166 ± 18	230 ± 58	12 ± 1	15 ± 4	67 ± 8	el	5 ± 0.58	el
		N	8	7	8	7	8	7	8	7
3	140 Days on DDT including 10 days' starvation followed by 40 days' recovery	Range	207–229	91–153	15–17	7–11	78–87		6–7	
		Mean ± S.E.	216 ±3	125 ±8	16 ± 0.51	9 ± 0.75	83 ± 1	el	6 ± 0.26	el
		N	6	6	6	6	6	6	6	6
4	140 Days on DDT, no starvation	Range	85–380	148–224	6–28	11–17	26–320	56–114	2–24	4–9
		Mean ± S.E.	287 ± 44	169 ± 11	21 ± 3.0	13 ± 1	170 ± 45	80 ± 16	13 ± 3.0	6 ± 1
		N	6	6	6	6	6	6	6	6

* el = Below the experimental limits of the method.

TABLE 6

DDT AND DDE IN THE LIVER OF RATS FED 200 PPM DDT IN THEIR DIET

Group number	History	Statistical evaluation	DDT (ppm)				DDE (ppm)			
			Lipids		Tissue		Lipids		Tissue	
			Male	Female	Male	Female	Male	Female	Male	Female
1	90 Days on DDT, prestarvation	Range	73–156	143–281	2–6	0.5–10	24–36	36–98	1–1	1–3
		Mean ± S.E.	116 ± 12	210 ± 23	4 ± 0.5	4 ± 1.0	31 ± 2	70 ± 8	1 ± 0	2 ± 0.3
		N	6	6	6	6	6	6	6	6
2	100 Days on DDT including last 10 days on starvation	Range	265–468	548–800	8–15	17–23	42–150	116–281	1–5	4–8
		Mean ± S.E.	349 ± 24	636 ±49	11 ± 0.9	19 ± 1.0	72 ± 12	202 ± 35	2 ± 0.4	6 ± 0.9
		N	9	5	9	5	9	5	8	5
3	140 Days on DDT including 10 days' starvation followed by 40 days on recovery diet	Range	130–316	210–479	4–11	7–17	27–64	30–183	1–6	1–7
		Mean ± S.E.	216 ± 25	370 ± 41	8 ± 0.9	13 ± 1	39 ± 5	121 ± 25	2 ± 0.8	4 ± 0.3
		N	6	6	6	6	6	6	6	6
4	140 Days on DDT, no starvation	Range	223–356	399–818	8–14	12–30	33–54	71–264	1–2	3–10
		Mean ± S.E.	282 ± 14	580 ± 89	11 ± 0.6	19 ± 3.2	47 [illegible]	145 ± 34	2 ± 0.2	5 ± 1.0
		N	8	6	8	6	[illegible]	6	8	6

TABLE 7

DDT and DDE in the Kidney of Rats Fed 200 ppm DDT in Their Diet

Group number	History	Statistical evaluation	DDT (ppm)				DDE (ppm)			
			Lipids		Tissue		Lipids		Tissue	
			Male	Female	Male	Female	Male	Female	Male	Female
1	90 Days on DDT, prestarvation	Range	527–666	595–1772	14–17	10–29		261–886		4–14
		Mean ± S.E.	586 ± 24	1114 ± 139	16 ± 0.5	18 ± 2	el[a]	304 ± 90	el	8 ± 2
		N	5	7	5	7	5	7	5	7
2	100 Days on DDT including last 10 days on starvation	Range	1149–3074	1164–3598	11–49	19–58	605–2735	392–1943	10–44	6–31
		Mean ± S.E.	2137 ± 221	2240 ± 335	33 ±4	35 ± 5	1580 ± 248	1172 ± 214	25 ± 4	19 ± 3
		N	8	8	8	8	8	8	8	8
3	140 Days on DDT including 10 days' starvation followed by 40 days' recovery	Range	978–2070	1877–3628	17–33	30–58	652–1229	1139–2008	10–20	2–32
		Mean ± S.E.	1415 ± 169	2581 ± 235	24 ± 2	41 ± 1	853 ± 106	1469 ± 119	15 ± 2	20 ± 1
		N	6	6	6	6	6	6	6	6
4	140 Days on DDT, no starvation	Range	380–668	962–2508	6.2–11.0	15–40		235–526		4–8
		Mean ± S.E.	534 ± 48	1518 ± 224	9 ± 1	24 ± 4	el	386 ± 42	el	6 ± 1
		N	6	6	6	6	6	6	6	6

[a] el = Below the experimental limits of the method.

liver (Table 6), and kidney (Table 7) of the same animals fed DDT at a rate of 200 ppm were more complicated than those for fat and plasma. In the brain, liver, and kidneys of both males and females, there was a significant increase in the concentration of DDT following limited starvation. However, interpretation of this fact was obscured by the further fact that storage after 140 days of DDT intake (groups 4M and 4F) was significantly greater in the liver of both sexes and in the brain of males than it was after only 90 days at the same rate (groups 1M and 1F), with no starvation in either instance. It therefore seems that the concentration of DDT in the liver of both sexes and in the brain of the males had not reached a steady state during 90 days of feeding—nor is there, of course, any evidence that a steady state had been reached even after 140 days.

The results for the storage of DDE in liver of both sexes and in the brain of males were essentially parallel to those for DDT. The fact that no DDE was found in the organs of some groups probably does not indicate its complete absence. Under the conditions of analysis, the concentration of DDE must be at least 10% that of DDT in order to be reported.

All the foregoing section on storage is concerned with rats that were killed for study according to a predetermined schedule. The results of analyses on animals that had died spontaneously are not strictly comparable to others because, among other things, the dying animals had undergone an uncontrolled, highly variable degree of starvation prior to death. It is of interest, however, to examine the concentration of DDT and DDE in the brain in relationship to symptomatology. Table 8 shows that the concentration of both DDT and DDE in the brain cor-

TABLE 8

DDT AND DDE STORAGE IN BRAIN LIPID OF RATS THAT DIED SPONTANEOUSLY DURING THE 10-DAY PERIOD OF STARVATION AND OF OTHERS THAT WERE KILLED AT THE END OF THE SAME PERIOD

Group	Number of rats	Clinical signs	Concentration (ppm)			
			DDT		DDE	
			Range	Mean	Range	Mean
3M	2	Died	205–985	595	78–374	226
2M	8	None	90–236	164	30–102	67
3F	2	Died	722–806	764	169–318	244
2F	4	Tremor	140–523	264	31–165	88
2F	3	None	124–126	125	32–37	34

responded closely with clinical state, reaching their highest concentration in animals that apparently had died of poisoning.

Excretion

The urinary excretion of DDA in 2 groups of rats that were starved while receiving DDT at a dietary level of 200 ppm is shown in Fig. 2A.

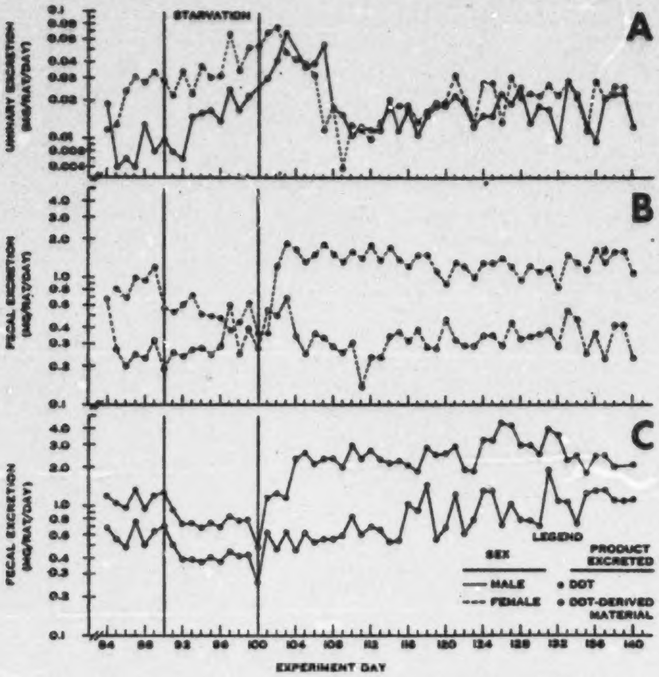

Fig. 2. Urinary excretion (A) and fecal excretion (B and C) of DDT and its derivatives in rats fed DDT.

These groups and 2 others that were killed at the end of the starvation period showed a gradual increase in excretion of DDA during the period of starvation even though their DDT intake was reduced to half. The rats that were permitted to resume feeding ad libitum continued to show an increase in excretion of DDA for about 2 days in the females and about 7 days in the males after starvation was stopped. Following the rise, excretion in the males and females decreased rapidly, reaching its lowest point in an additional 3–7 days. Urinary excretion then increased gradually, reaching a steady state about 20 days after starvation stopped.

Fecal excretion of DDT-derived material in 2 groups of rats under discussion is shown in Fig. 2B for females and in Fig. 2C for males. (The results for rats killed at the end of the starvation period were similar.) For each group, the lower curve represents the excretion of metabolized DDT reported as DDA and the upper curve represents the passage of unchanged DDT. Other studies to be reported elsewhere offer adequate evidence that DDT in the feces represents unabsorbed material that has been carried mechanically by the fecal stream. In both sexes the "excretion" of DDT corresponded with food intake, and therefore, with DDT dosage.

The curve for true fecal excretion (Fig. 2B) in the females was essentially similar in its time relationships to that for urinary excretion (Fig. 2A), although the degree of change during starvation and the decrease shortly after starvation was less marked in the case of fecal excretion. The curve for true fecal excretion in the males apparently did not show the same pattern. Fecal excretion of DDA-like material did not show an absolute increase in males during starvation; however, it did not undergo quite as much reduction as the dosage and, therefore, showed a slight relative increase.

During the steady state of storage resulting from intake of food containing DDT at a concentration of 200 ppm, male rats excreted daily more metabolized DDT in the urine and feces combined either on a milligram per rat or a milligram per kilogram basis, but this difference is further increased when excretion is expressed on the basis of milligram per kilogram per 1000 ppm of DDT-derived material stored in the fat (Table 9). The difference is somewhat greater in each of the three comparisons if dosage is taken into account because the females ate 1.15 times more food than the males in proportion to their weight. Thus, for any given level of DDT storage, the males were more efficient in excretion. There was, however, some evidence from a graph (not shown) of excretion in terms of body weight and also from a graph of excretion expressed as a ratio of storage that the superiority of the male in excretion was less marked during starvation than at other times. In fact, the same conclusion is supported by the failure of the curve for DDA to rise during the starvation period in Fig. 2C.

TABLE 9

MEAN DAILY VALUES FOR STEADY STATE EXCRETION OF METABOLIZED DDT IN MALE AND FEMALE RATS (120TH TO 140TH DAY OF EXPERIMENT)

Unit	Males	Females
Milligrams per rat per day	1.10	0.39
Milligrams per kilogram of body weight per day	3.01	1.88
Milligrams per kilogram of body weight per day per 1000 ppm of DDT-derived material stored in fat	3.86	0.99

DISCUSSION

A study of partial starvation accompanied by continued, though reduced, DDT dosage was considered important because (1) it is more likely to occur in people than is complete starvation with complete cessation of DDT intake, and (2) it provides a more severe test as evidenced by the occurrence of tremors in female rats on a dosage level too low to produce typical signs in connection with complete starvation. It may be mentioned that the brain levels of DDT that were produced by repeated dosage and were associated with tremor and survival or with tremor and death were of the same order of magnitude as the levels produced by a single large dose and associated with the same degrees of illness. This is consistent with the view that the tremors and other signs of illness are a direct response to the concentration of DDT in the brain at the moment.

The observation that a steady state of storage was not reached in the brain, liver, and kidney of one or both sexes after 90 days of DDT intake at a dietary rate of 200 ppm is unexplained and requires further study. It is difficult to see how a steady state could exist in some tissues (fat and plasma) unless it existed in all tissues. The difficulty of analysis of DDT-derived material in brain, liver, and kidney is greater than that of analysis of the same material from fat. This fact must be considered in evaluating the apparent lack of equilibrium.

The results reported are consistent with the assumption already made by several investigators and reviewed elsewhere (Hayes, 1959) that most of the DDT in the body is stored chiefly in neutral fat and that, when this fat is used up during starvation, the concentration of DDT and DDE in the remaining adipose tissue (still largely neutral fat) is increased. During starvation the concentrations of DDT and DDE apparently increase in the other tissues also, so that an equilibrium is maintained.

It appears clear that the rate of true excretion of DDT-derived material is more immediately related to storage, particularly the concentration in the plasma, than to dosage. Although no measurement was made of tissue levels at intervals after starvation was stopped, it seems probable that the general form of the excretion curves can be explained by taking DDT dosage and the recovery of neutral fat stores into account. The continued increase of excretion immediately after starvation was stopped corresponded to a period of high DDT dosage. The subsequent decrease in excretion corresponded with a decrease in the food intake and a further increase in body weight and must have corresponded with a relatively rapid restoration of the fat stores. The excretion then began to approach equilibrium at the same time that the food intake, dosage, and body weight of the rats leveled off.

The findings of this study both in regard to storage and excretion help to explain the relative susceptibility to DDT poisoning of animals that are emaciated. The susceptibility of fish, birds, and mammals as related to nutritional state has been reviewed elsewhere (Hayes, 1959).

This study does not support the view that DDT presents a hazard to people subject to starvation (whether as the result of dieting, deprivation, or disease) because the concentration of DDT in men with the greatest occupational exposure (Mattson *et al.*, 1953; Ortelee, 1958)—not to mention people of the general population (Hayes *et al.*, 1958)—is much less than that necessary to produce the phenomenon in experimental animals. Furthermore, the metabolism of man is slower than that of the rat, so that the phenomenon would be less likely to occur in man even at the same concentration of DDT storage.

SUMMARY

Before and immediately after a 10-day period of partial starvation and again after 40 additional days' recovery, DDT and its metabolites were measured in the fat of rats fed traces of the insecticide and in the fat, plasma, brain, liver, kidney, urine, and feces of rats fed the compound at a rate of 200 ppm. Mobilization of body fat during starvation resulted in an increased concentration of DDT-derived material in that tissue and a corresponding increase in the other tissues. An augmented excretion of metabolites occurred during starvation in spite of decreased intake of DDT. The increased excretion was inadequate to prevent the increase in concentration of DDT-derived material in the body although it tended to do so. When sufficiently

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great, the augmented concentration of DDT in the brain associated with starvation was correlated with the occurrence of signs of poisoning. Storage and excretion tended to return to prestarvation levels during recovery, but some lack of parallelism was observed between the concentration of DDT-derived materials in brain, liver, and kidney as compared with fat and plasma.

REFERENCES

Bloor, W. R. (1914). A method for the determination of fat in small amounts of blood. *J. Biol. Chem.* **17**, 377-384.

Fitzhugh, O. G., and **Nelson**, A. A. (1947). The chronic oral toxicity of DDT (2,2-bis[*p*-chlorophenyl]-1,1,1-trichloroethane). *J. Pharmacol. Exptl. Therap.* **89**, 18-30.

Hayes, W. J., **Jr.** (1959). Pharmacology and toxicology of DDT. In *The Insecticide Dichlorodiphenyltrichloroethane and Its Significance* (P. Müller, ed.), Vol. II, Chapter VI, pp. 9-247. Birkhäuser, Basel.

Hayes, W. J., **Jr.**, **Quinby**, G. E., **Walker**, K. C., **Elliott**, J. W., and **Upholt**, W. M. (1958). Storage of DDT and DDE in people with different degrees of exposure to DDT. *A.M.A. Arch. Ind. Health* **18**, 398-406.

Jensen, J. A., **Cueto**, C., **Dale**, W. E., **Rothe**, C. F., **Pearce**, G. W., and **Mattson**, A. M. (1957). Metabolism of insecticides, DDT metabolites in feces and bile of rats. *J. Agr. Food Chem.* **5**, 919.

Mattson, A. M., **Spillane**, J. F., **Baker**, C., and **Pearce**, G. W. (1953). Determination of DDT and related substances in human fat. *Anal. Chem.* **25**, 1065-1070.

Ofner, R. B., and **Calvery**, H. O. (1945). Determination of DDT (2,2-bis[*p*-chlorophenyl]-1,1,1-trichloroethane) and its metabolite in biological material by use of the Schechter-Haller method. *J. Pharmacol. Exptl. Therap.* **85**, 363-370.

Ortelee, M. F. (1958). Study of men with prolonged intensive occupational exposure to DDT. *A.M.A. Arch. Ind. Health* **18**, 433-440.

Schechter, M. S., **Soloway**, S. B., **Hayes**, R. A., and **Haller**, H. L. (1945). Colorimetric determination of DDT. *Ind. Eng. Chem. Anal. Ed.* **17**, 704-709.

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TOXICOLOGY PROGRAM

HEXANE EXTRACTABLE CHLORINATED INSECTICIDES IN HUMAN BLOOD

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It is generally agreed, on the basis of pharmacological evidence, that the most important site of action of chlorinated insecticides is the central nervous system. This was confirmed in connection with DDT by the fact that the severity of clinical signs of poisoning is directly proportional to the concentration of the unmetabolized compound in the brain (1) and by the fact that the concentration in the brain which leads to death is of the same order of magnitude no matter whether animals are killed by a single large dose or by many small doses (2). Much valuable information may be obtained from analysis of chlorinated hydrocarbon insecticides in the body fat of people in the general population or in any other group whose manner of exposure to the compounds is known. However, in cases in which the exposure is not known, fat is not a satisfactory tissue for analysis, and it is just such cases that are most likely to involve poisoning as a differential diagnosis. The concentration of DDT in the fat, unlike that in the brain, bears no necessary relation to the presence or degree of poisoning (2). Furthermore, samples of fat may be difficult to obtain from some people one would like to study. Samples of human brain are available from some routine autopsy material and may be available in rare cases in which poisoning by a pesticide is suspected as the cause of death, but brain samples are completely unavailable for most diagnostic study or for meaningful monitoring.

Unlike fat or especially brain, blood can be obtained conveniently from almost anyone. There is some evidence that the concentration of DDT in blood tends to correspond to that in the brain and to show far less relation to the concentration in the fat (3). It has also been pointed out that the concentration of a drug at receptor sites is related to the concentration of unbound drug in the plasma (4). Because of its availability and probable diagnostic value, blood would seem to be a good tissue in which to measure

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the concentration of chlorinated hydrocarbon insecticides, and therefore it was studied in this connection.

Several methods have been developed in recent years for the determination of chlorinated insecticides in human blood utilizing gas chromatography and highly sensitive detectors (5, 6, 7). The method described by Robinson (5) utilized alkaline saponification as a clean-up procedure and therefore was unsuitable for the determination of insecticides that are unstable to alkali. The method of Schmit *et al.* (6) for the determination of DDE, o,p'-DDD, and p,p'-DDT was unsatisfactory in that interfering materials produced multiple large peaks in the early part of the chromatograms where the peaks for several chlorinated insecticides or related materials would also appear. The method described by Kadis and Jonasson (7) is not only time consuming but lacks the sensitivity required for the analysis of these materials in blood of the general population. A rapid, simple, and sensitive procedure for the determination of chlorinated insecticides and related materials in blood of the general population was not available. Since such a method is essential if analysis of blood is to be used for the toxicological evaluation of human exposure to insecticides, the following procedure was developed.

Method

Ten milliliter samples of blood were collected from each subject. Ground glass stoppered centrifuge tubes were used throughout. About 4 ml of each sample were placed in a 15-ml centrifuge tube and allowed to clot. The remaining 6 ml of blood were put in another centrifuge tube containing 0.1 ml of 20% aqueous potassium oxalate as an anticoagulant. After the contents of the tube were mixed gently by inversion, 2 ml of the oxalated whole blood were pipetted into a 15-ml centrifuge tube and held for analysis. The remaining oxalated blood was centrifuged at 2000 rpm for 10 minutes in order to separate the plasma from the red blood cells (RBC). The sample of clotted blood was centrifuged in a similar manner to remove the clot from the serum. Two ml of plasma, RBC, and serum were pipetted into separate 15-ml centrifuge tubes, and 5 ml of nanograde n-hexane were added to each of these subsamples as well as to the subsample of whole blood previously measured out. The 4 subsamples were then shaken for 3 minutes with the aid of a mini-mixer. When emulsification occurred during this extraction procedure, the emulsions were broken by centrifugation at 2000 rpm for 5 minutes. The lower aqueous phase was then removed from each tube by a capillary pipette and discarded. Each n-hexane extract was dried by adding a small amount of anhydrous Na_2SO_4 and shaking the tube on the mini-mixer. A 4-ml aliquot equivalent to 1.6 ml of each original subsample was then pipetted into a 15-ml centrifuge tube, and the tubes were placed in a 40° C constant temperature water bath. The solvent was removed with the aid of a gentle stream of clean dry air. The residue was taken up in 50 µl of nanograde hexane, and an aliquot of 10 µl equivalent to 0.32 ml of the subsample was subjected to gas liquid chromatography.

A MicroTek MT-220 gas chromatograph equipped with an electron capture detector and a strip chart recorder with a disc integrator was used. In order to confirm the identity of the materials in the effluent gas, two kinds of columns were used. Column No. 1 consisted of an aluminum tube, 6 feet x 3/16 inch, O.D., packed with 10% diethylene glycol succinate (D.E.G.S.) on 60/80 mesh, acid washed chromosorb-G. Column No. 2 consisted of an aluminum tube, 4 feet x 1/4 inch O.D., packed with 5% Dow 200 oil on 60/80 mesh acid washed chromosorb-G. Both columns were operated under the following conditions: inlet and outlet blocks 224° C; column oven, 180° C; detector, 185° C; carrier gas, N_2 80 cc/minute at 40 psi; scavenger gas, N_2 250 cc/minute at 40 psi; power source, direct current at 15 volts. The retention times in minutes for Column No. 1 and Column No. 2, respectively, were as follows: p,p'-DDT, 30.3 and 38.2; o,p'-DDT, 16.0 and 28.7; p,p'-DDE, 11.3 and 22.0; o,p'-DDE, 8.4 and 17.8; p,p'-DDD, 38.3 and 28.7; alpha-BHC, 4.4 and 4.2; beta-BHC, 23.8 and 4.6; gamma-BHC, 7.0 and 5.2; delta-BHC, 21.0 and 5.2; aldrin, 3.1 and 11.3; dieldrin, 14.4 and 22.0; and heptachlor epoxide, 9.6 and 14.4.

Results

All of the isomers and metabolites just listed except aldrin and delta-BHC have been found in the fat of persons without occupational exposure to insecticides (8,9) with the further exception that so far alpha-BHC has been reported only from India (8) and from England and Wales (9), and gamma-BHC has been reported only from England and Wales (9). Thus, it is reasonable to think that 8 isomers or metabolites of chlorinated hydrocarbon insecticides might be detectable in the blood of Americans who have no special exposure to insecticides. Actually, 6 of them (p,p'-DDT, o,p'-DDT, p,p'-DDE, beta-BHC, dieldrin, and heptachlor epoxide) were found in measurable quantity in the blood of 10 men with no occupational exposure to insecticides as shown in Table 1, which is based on results obtained with Column No. 1. Results with Column No. 2 confirmed the identity of the 6 compounds and were essentially identical from a quantitative standpoint. All of the other compounds, whether demonstrated in fat or not, would have been detected if they had been present in the subsamples of blood at a concentration of 0.0001 ppm or more.

If, as an approximation, one considers the hematocrit to be 50%, it can be readily seen that the concentration of chlorinated insecticides and related materials found in whole blood is in reasonably good agreement with the total concentrations found in plasma and RBC separately, i.e., (plasma + RBC)/2 = whole blood. As shown in Table 1, 45% or more of each n-hexane extractable insecticide and related material in the blood was found in the serum. Since there was no significant difference ($p > 0.2$) between the concentrations of insecticides or related materials found in plasma and those found in serum, most further studies were limited to analysis of serum.

TABLE 1

Concentration and Distribution of Chlorinated Insecticides and Related Materials in Ten Human Blood Samples as Determined by Electron Capture, Gas Liquid Chromatography

	p,p'-DDT	o,p'-DDT	p,p'-DDE	beta-BHC	Dieldrin	Heptachlor Epoxide
Whole Blood						
Range (ppm)	0.0024- 0.0106	0.0005- 0.0017	0.0045- 0.0235	0.0019- 0.0042	<0.0001- 0.0028	0.0004- 0.0019
Mean	0.0058	0.0010	0.0114	0.0031	0.0014	0.0008
S.E.	± 0.0009	± 0.0002	± 0.0021	± 0.0002	± 0.0002	± 0.0002
RBC						
Range (ppm)	0.0008- 0.0049	0.0002- 0.0006	0.0024- 0.0137	0.0005- 0.0022	<0.0001- 0.0009	0.0004- 0.0010
Mean	0.0026	0.0004	0.0063	0.0010	0.0005	0.0007
S.E.	± 0.0004	± 0.00004	± 0.0012	± 0.0002	± 0.0001	± 0.0001
Plasma						
Range (ppm)	0.0034- 0.0305	<0.0001- 0.0036	0.0069- 0.0499	0.0015- 0.0062	0.0009- 0.0037	0.0002- 0.0031
Mean	0.0119	0.0013	0.0257	0.0034	0.0019	0.0011
S.E.	± 0.0025	± 0.0004	± 0.0043	± 0.0005	± 0.0003	± 0.0003
Serum						
Range (ppm)	0.0042- 0.0290	0.0003- 0.0014	0.0055- 0.0475	0.0004- 0.0094	0.0003- 0.0032	0.0006- 0.0028
Mean	0.0105	0.0009	0.0198	0.0036	0.0017	0.0012
S.E.	± 0.0023	± 0.0001	± 0.0041	± 0.0008	± 0.0003	± 0.0002
Plasma vs. Serum	p>0.5	p>0.2	p>0.5	p>0.5	p>0.5	p>0.5
Proportion in Serum (%)*	90	45	87	58	61	75

* $\frac{1}{2} \times \frac{\text{serum}}{\text{whole blood}} \times \frac{100}{1} = \%$

In order to determine the efficiency of the method, standard materials in amounts approximately 100 times those found in 1 ml of serum were added in n-hexane to each of six 15-ml centrifuge tubes. The factor of 100 was used so that the concentrations present in the serum subsequently added could be ignored in the calculations. The hexane was evaporated just to dryness with a gentle stream of clean dry air. To each tube was added a 1-ml aliquot of the same pooled serum, and the contents were mixed several times, using a mini-mixer, over a period of 2 hours. Three of the samples were extracted as described in the method and three samples were given three successive extractions. Data presented in Table 2 show that there were no significant differences in recovery between single and triple extractions ($p > 0.2$ in each instance) and that these recoveries ranged from a low of 29% for aldrin to a high of 91% for dieldrin.

TABLE 2

Recovery* of Various Chlorinated Insecticides and Related Materials Added to 1 ml Serum. Comparison of Single vs. Triple Extraction.

		Extractions			
	Micrograms	Single		Triple	
Material	Added	%	S.E.	%	S.E.
p,p'-DDT	3.237	45	10.2	42	11.9
o,p'-DDT	1.082	37	9.7	37	10.0
p,p'-DDE	3.235	58	9.5	67	9.2
alpha-BHC	1.015	85	2.4	74	9.5
beta-BHC	1.058	70	2.0	66	9.9
gamma-BHC	1.029	85	2.0	73	11.0
delta-BHC	1.116	76	1.8	69	12.0
Aldrin	0.108	29	9.6	26	7.2
Dieldrin	0.098	91	5.9	76	17.3
Heptachlor epoxide	0.105	82	4.5	67	17.3

*Mean of three determinations.

The failure of succeeding extractions to improve recovery indicates _in vitro_ interaction or binding of the added insecticides with some constituent of serum. The possibility of further _in vitro_ binding during storage of the insecticides normally present in human blood was investigated. Three samples of serum were collected and aliquots taken for analysis that was completed within 2 hours. The remaining portions of the samples were stored in a refrigerator and aliquots again analyzed at the end of 5 and 7 days. There was no significant difference detected between the 2 aliquots of each sample due to storage.

The method was applied to persons who have been occupationally exposed to aldrin and dieldrin over a period of 8 years but who have not shown any clinically detectable signs of poisoning. Exposure ratings of high, medium, and low were assigned based on hours of exposure. The data presented in Table 3 show that the concentrations of aldrin and dieldrin in plasma correspond to the extent of exposure.

TABLE 3

Average Concentration of Aldrin and Dieldrin in the Plasma of Occupationally Exposed People

Exposure Rating	Mean Exposure (hours)	Persons Exposed	Aldrin (ppm)	Dieldrin (ppm)
High	6044	6	0.0023	0.0270
Medium	1410	8	0.0015	0.0206
Low	259	10	0.0007	0.0094

Plasma collected from an adult male (061229 ES 21)* about 18 hours after he ingested aldrin and about 10 hours after the last convulsion was found to contain 0.0360 ppm of aldrin and 0.279 ppm of dieldrin. Twenty days later the plasma concentration of aldrin and dieldrin had decreased to 0.0018 ppm and 0.090 ppm, respectively (10).

Plasma taken from a boy (071963 JW 24) approximately 6 hours after he swallowed lindane (99% gamma-isomer of BHC) and 3 hours after the last convulsion was found to contain gamma-BHC at a concentration of 0.29 ppm. Seven days following ingestion of lindane the patient was asymptomatic and the plasma concentration of gamma-BHC had decreased to 0.02 ppm (10).

Discussion

In order to maintain a base-line variation of less than 1% of full scale under the conditions described in this paper, the attenuation of sensitivity settings of the instrument were such that the minimal detectable amount of any of the chlorinated insecticides studied was 0.04 nanograms. This resulted in a need to concentrate the n-hexane extract of samples from the general population (but not from those with added material) in order to be able to inject 10 µl of extract and yet be able to report results as low as 0.0001 ppm.

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*Cases are identified by date of birth, first and last initials, sex, and race according to the conventions used by the AMA Council on Drugs, Registry on Adverse Reactions, for reporting adverse reactions to drugs and other chemicals.

With reagent blanks, the procedures used in this study did not give an elevated base line nor any peaks within the pertinent range of the chromatogram. The small amount of sodium sulfate used gave a peak only beyond the pertinent range of the chromatogram and, therefore, did not interfere with the determination of any of the compounds studied. It should be pointed out that certain plastic materials, florisil, alumina, and glass distilled water contained interfering materials that became very apparent at the sensitivity required for this procedure.

The possibility of recovering materials added to blood depends upon the manner in which they are added. If an organic solvent is added to the blood prior to addition of the standard materials or if a solution of the standard materials is added to the blood, quantitative recoveries are obtained. However, if the blood or blood fraction is added to a dry residue of the standard materials, as described in this paper, a single n-hexane extraction gives recoveries ranging from 27% for aldrin to 91% for dieldrin. Residues of all of the insecticides can be recovered quantitatively from glass in the absence of serum irrespective of the presence of water. The fact that successive extractions produced no improvement in the recovery of any of the insecticides demonstrates binding of these materials with some constituent of serum, e.g., lipoproteins. This in vitro binding indicates possible in vivo binding. Therefore, it may be that n-hexane extracts not the total but only the free (unbound) insecticides and related materials present in serum.

As already mentioned, it has been shown (4) that the concentration of the unbound rather than the bound foreign chemicals in plasma correlates with the intensity and duration of effect at the site of action. The concentration of each of the n-hexane extractable insecticides and related materials present in plasma (Table 3) correlates with the extent of exposure to chlorinated insecticides. Furthermore, the concentrations of dieldrin (Table 1) found in the general population by use of this method are in agreement with those obtained by Robinson (5) by use of a more lengthy procedure which was reported to give quantitative recoveries of materials that were added, albeit in solution.

Kadis and Jonasson (7) abandoned the analysis of plasma or serum for that of whole blood because insecticides are present in the red blood cell fraction also. As shown in Table 1, the relative amount of insecticides or related materials in RBC and in plasma varies depending upon the material considered. However, in all cases these materials in the blood are present in substantial concentration in the serum. It may be desirable in some studies to determine the concentration of the n-hexane extractable materials in whole blood or in each fraction of blood separately. However, for most toxicological evaluations in which the extent of exposure to insecticides is to be evaluated, the determination of the level of these materials in plasma or serum would be satisfactory.

Although no aldrin or gamma-BHC was found in any of the people of the general population, aldrin was found in the plasma of occupationally and acutely exposed persons, and gamma-BHC was found in the plasma of a boy who swallowed it. The occupationally exposed, asymptomatic persons had only 6% to 18% of the "threshold for dieldrin intoxication" (0.15 ppm) suggested by Brown et al. (11). The individual who swallowed aldrin had 0.036 ppm aldrin and 0.279 ppm dieldrin in his plasma at the time he had characteristic signs of poisoning. During gradual recovery, the plasma concentration of aldrin and dieldrin decreased to 0.0018 ppm and 0.090 ppm, respectively. This again correlated well with the threshold suggested by Brown et al. (11).

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The method described has detected in blood 6 of the 8 isomers or metabolites of chlorinated hydrocarbon insecticides known to occur in the fat of people in the general population of the United States, and it has detected 2 others in people with occupational or acute exposure. Although low recoveries are obtained of added materials apparently due to binding on some constituents of blood, this may offer some advantage. In any event, the concentration of chlorinated insecticides and related materials as detected by this method follows the pattern of exposure and recovery and thus permits toxicological evaluation.

REFERENCES

1. W.E. DALE, T.B. GAINES, W.J. HAYES, JR. and G.W. PEARCE, Science 142, 1474 (1963).
2. W.J. HAYES, JR. and W.E. DALE, Toxicol. Appl. Pharmacol. 6, 349 (1964).
3. W.E. DALE, T.B. GAINES and W.J. HAYES, JR., Toxicol. Appl. Pharmacol. 4, 89 (1962).
4. B.B. BRODIE, G.J. COSMIDES and D.P. RALL, Science 148, 1547 (1965).
5. J. ROBINSON, Proc. Third International Meeting in Forensic Immunology, Medicine, Pathology and Toxicology, London, 1963, to be published.
6. J.A. SCHMIT, R.B. WYNNE and U.J. PETERS, Biomedical GC Notes, F. and M. Corporation, Avondale, Pa. (1964).
7. V.W. KADIS and O.J. JONASSON, 56th Annual Meeting of the Canadian Public Health Association, Edmonton, Alberta (1965).
8. W.J. HAYES, JR., W.E. DALE and V.W. BURSE, Life Sci. 4, 1611 (1965).
9. H. EGAN, R. GOULDING, J. ROBURN and J. O'G. TATTON, Brit. Med. J. 2, 66 (1965).
10. W.E. DALE, G.E. QUINBY, W.F. DURHAM and A. CURLEY, In preparation.
11. V.K.H. BROWN, C.G. HUNTER and A. RICHARDSON, Brit. J. Industr. Med. 21, 283 (1964).

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Penetration of the Blood-Brain-Cerebral Spinal Fluid Barrier by DDT

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The central nervous system (CNS) is a unique organ system in that it not only receives a rich vascular supply, but, in addition, it is suspended in the cerebral spinal fluid (CSF). Basic toxicological information about any neurotoxic compound includes ascertaining the absolute and relative amounts of the compound in these three separate, but intimately related, compartments (blood, CSF, brain) at a given time after the administration of an intoxicating dose of the compound. The present study was undertaken to obtain this information for the organochlorine compound, p,p'-DDT (2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane).

The chief manifestation of DDT intoxication involves central nervous system dysfunction (1). However, the mechanism by which DDT effects these aberrations remains unknown. Before it can be elucidated, the accessibility of the compound to, and its affinity within, the CNS must be established.

There have been reports dealing with DDT concentrations in blood and brain after a single oral dose in the rat (2-4), a single intravenous dose in cats (5), and after long-term daily dosing in rats (6,7). Apparently, however, no data have been published of the concentration of DDT in CSF.

Materials and Methods

Twenty-four Sprague-Dawley weanling rats, random-bred, were housed individually and fed ground chow and water *ad libitum* until adulthood (90-120 days of age). At that time they were divided into three groups of equal sex distribution.

The rats were dosed orally by gastric intubation with 200, 400, or 600 mg/kg of analytical grade (99.9% pure) p,p'-DDT dissolved in warmed peanut oil (38°C). The dose was administered on a fixed volume per weight basis (0.005 ml of solution per g of body weight) according to the method of Gaines (8). Doses above the LD_{50} of DDT (8) were used to facilitate the development of neurotoxic signs.

The animals were observed until generalized convulsive activity occurred; at this time CSF was collected. The animals were anesthetized with ether and placed in a special stand (9) or their head was acutely flexed over the edge of a table, and a No. 26 gauge needle was inserted into the cisterna magnum. Although it was difficult to obtain the necessary 0.02-0.10 ml of clear, colorless fluid before blood appeared, this was essential; if the fluid contained even a few red blood cells the amount of DDT was spuriously elevated.

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After the CSF was obtained, a 0.1-ml sample of blood was collected by cardiac puncture. The animal was then sacrificed and a 2-400 mg section of cerebral hemisphere was removed.

All samples were placed in pre-weighed glass tubes; sintered-glass hand tissue-grinding tubes were used for blood and brain and small centrifuge tubes were used for CSF. They were then extracted as follows.

CSF was extracted with 1 ml of hexane.

Two milliliters of distilled water was added to the blood sample, the mixture was extracted with 5 ml of hexane, and 1:5, 1:6, or other suitable dilutions were prepared in hexane (10).

Brain was extracted with 2.5 ml of acetonitrile, an equal volume of 2% aqueous sodium sulfate was added, and the mixture was then extracted three times with 2 ml of hexane. The extracts were combined and 1:10 or other suitable dilutions were made in hexane (11).

All analyses were performed with a Micro-Tek MT-220 gas-liquid chromatograph equipped with a Ni^{63} electron-capture detector. The 6-foot, 1/4-inch chromatographic column was packed with 1.95% QF-1 + 1.5% OV-17 coated on 80-100 mesh HP Chromosorb W. All samples were analyzed against a standard DDT solution of 133 pg per 10 μl. The temperature, voltage, and flow rates of nitrogen were adjusted to give a DDT retention time of approximately 15 min with maximal sensitivity. Picogram amounts were analyzed by this method. The peaks were sharp and symmetrical and no other significant peaks appeared. Under these conditions there was no detectable DDT in samples of CSF, blood, or brain from non-poisoned animals.

Results and Discussion

The means, ranges, and standard deviations of the p,p'-DDT concentrations are summarized in Table 1. The mean concentration in all dosed animals at the time of convulsion was 0.12 ppm in the CSF, 3.4 ppm in the blood, and 16 ppm in the brain. There was no significant sex difference in the levels attained under these experimental conditions. The mean DDT levels in these three tissues were also independent of the dosages given in this study.

All treated animals convulsed between 2 and 8 hours after dosing. Although there was no significant difference in the ultimate convulsing concentration of DDT in these three tissues, the animals given the higher doses (i.e., 600 mg/kg) developed signs of intoxication in the early part of this period (2-4 hr) and those given the lower doses (i.e., 200 mg/kg) developed signs in the later part of this period (5-8 hr).

TABLE 1

p,p'-DDT Concentrations in the Cerebral Spinal Fluid (CSF), Blood, and Brain of Rats at the Time of p,p'-DDT-Induced Convulsions[a]

p,p'-DDT Administered, mg/kg	p,p'-DDT Concentration, ppm					
	CSF		Blood		Brain	
	Male	Female	Male	Female	Male	Female
200	0.11(0.10-0.11)	0.13(0.11-0.14)	4.5(3.7-5.2)	3.7(2.8-4.6)	13(10-15)	17(16-17)
	0.12 ± 0.02		4.1 ± 1.0		15 ± 3	
400	0.17(0.15-0.18)	0.11(0.09-0.12)	4.6(3.9-5.3)	2.8(2.4-3.2)	13(12-14)	18(16-19)
	0.14 ± 0.04		3.7 ± 1.9		15 ± 3	
600	0.09(0.04-0.12)	0.14(0.12-0.16)	2.3(2.0-2.5)	3.0(2.4-3.6)	16(15-16)	18(18-19)
	0.11 ± 0.04		2.6 ± 0.6		17 ± 2	
200-600	0.12 ± 0.04	0.12 ± 0.02	3.6 ± 1.2	3.2 ± 0.8	14 ± 2	18 ± 1
	0.12 ± 0.03		3.4 ± 1.1		16 ± 3	

[a] The separate values for males and females are given as means and ranges of 4 rats; combined values are means ± SD of 8 rats, and 12 and 24 rats at all dosages.

The concentrations of DDT are expressed as ppm (µg/g) of tissue. Many investigators, e.g., Dale, et al. (2), have reported DDT levels in various body organs as ppm in tissue lipid. Although most of the DDT is held in the lipid fraction of a tissue, if that fraction constitutes varying amounts of the whole tissue, the measured concentration is then dependent upon two variables: the amount of lipid in the tissue and the amount of DDT in the lipid. Since the amount of lipid may not be constant in blood, it is perhaps preferable to express the DDT concentration as ppm of whole blood (or plasma). A slightly different argument has been advanced for expressing brain concentrations of DDT on a fresh weight basis and not on the basis of tissue lipid (3).

Nevertheless, DDT is lipophilic, it is concentrated and stored in adipose tissue, and, in general, the amount of DDT in any particular body tissue is a function of the tissue lipid content (1,12). However, other factors must also be considered, especially in a dynamic situation such as acute intoxication. Brain total lipid is about 12 g % of fresh tissue (grey matter 4.0-7.9 g %, white matter 13.9-23.1 g % (13,14), blood total lipid 650 mg % (450-900) (14,15), and spinal fluid 1-2 mg % (14,15). Thus, blood and brain have lipid concentrations approximately 400 and 8000 times that of CSF. If the concentration of DDT is solely a function of the amount of lipid in that tissue, then a similar proportional fractionation can be anticipated. But, in fact, the blood and brain DDT concentrations were only 30 and 130 times that found in the CSF. It would appear that under these experimental conditions the amount of DDT in the CSF was greater than that anticipated from its lipid content alone and/or the amount in the blood and brain was considerably less than that anticipated.

The latter possibility seems unlikely because the values reported here compare favorably to those previously reported for blood (3-5,16) and for brain (3-5). Therefore, the amount of DDT in the CSF of these animals is larger than that anticipated. The physiological significance of this is wholly speculative.

Most compounds enter the brain from the blood; in doing so they must first cross the "blood-brain barrier" (17) or the "blood-CSF barrier" (18). This concept of a "brain barrier system" (19) encompasses the partitioning of a compound in the blood, CSF, and brain compartments. It is a physiological barrier of pharmacological selection whose degree of development will depend on the species and the age of the animal (20). Compounds differ greatly in their ability to cross this "barrier" (18); in, general, the more lipid-soluble the compound, the more easily it can cross. Some compounds may exert their effect on nervous tissue via the CSF and/or the CSF may serve as a reservoir for the compound.

In clinical toxicology the concentration of a toxicant is measured in a readily accessible biological sample (e.g. blood) and then these data are extrapolated to the individual as a whole. Thus, with organochlorine compounds, attempts have been made to assess the effects in the end organ (the CNS) by measuring blood concentrations under various conditions.

In man, exposure to DDT is measured by levels in blood (21), but the latter are not necessarily related to clinical symptoms or signs. In rats, although the severity of signs of acute poisoning with DDT is directly proportional to the concentrations of the compound in their brains (2,7), concentrations in blood do not correlate with those in brain (4). This lack of correlation is in contrast to observations with dieldrin; in dogs fed a daily dose of the compound, the concentration of dieldrin in the blood and the severity of the clinical signs of intoxication were directly related (22). This apparent contradiction between two organochlorine compounds may not be real. There is a differential distribution of DDT, related to time after exposure, within the various areas of the CNS (4,5); with repeated dosing an equilibrium may be established that is not established in an acute intoxication.

Therefore, exposure can be related to concentrations in blood and these concentrations are not predictive of the concentrations in brain (at least in acute intoxications); however, the concentrations in brain are directly related to clinical signs of intoxication. Where the CSF should be fitted into this scheme is not readily apparent and in the present study a particular sign of intoxication (namely convulsions) was used as the end point. It is interesting to speculate that the CSF might subserve an important transport and/or storage role in the dynamics of DDT intoxication.

Summary

Adult male and female rats were orally dosed with 200, 400, or 600 mg/kg of p,p'-DDT. When convulsions occurred, samples of cerebral spinal fluid, blood, and brain were collected and assayed for p,p'-DDT. Means of 0.12, 3.4, and 16 ppm, respectively, were found. Neither the sex of the animal nor the dosage given affected the concentrations present in these three tissues at the time of convulsions.

References

1. HAYES, W.J., JR., in DDT, The Insecticide Dichlorodiphenyltrichlorethane and Its Significance, vol. II, P. Muller, Ed., pp 9-247 (1959), Birkhauser, Basel.
2. DALE, W.E., GAINES, T.B., HAYES, W.J., JR., and PEARCE, G.W., Science, 142, 1474 (1963).
3. WOOLLEY, D.E., and RUNNELLS, A.L., Toxicol. Appl. Pharmacol. 11, 389 (1967).
4. SANTOLUCITO, J.A., CUETO, C., WHITCOMB, E., and OWENS, J., Toxicol. Appl. Pharmacol. 14, 621 (1969).
5. SCHWABE, U., Naunyn Schmeidebergs Arch. Exp. Pathol. Pharmakol., 250, 84 (1965).
6. DALE, W.E., GAINES, T.B., and HAYES, W.J., JR., Toxicol. Appl. Pharmacol. 4, 89 (1962).
7. HAYES, W.J., JR., and DALE, W.E., Toxicol. Appl. Pharmacol. 6, 349 (1964).
8. GAINES, T.B., Toxicol. Appl. Pharmacol. 2, 88 (1960).
9. JEFFERS, W.A., GRIFFITH, J.Q., JR., and ROBERTS, E., in The Rat in Laboratory Investigation, 2nd ed., E.J. Farris and J.Q. Griffith, Jr., Eds., p 196 (1949), Hafner, New York.
10. RADOMSKI, J.L., and FISFROVA-BERGEROVA, V., Indust. Med. Surg. 34, 934 (1965).
11. "Micro Method for Human Tissue", in Pesticide Analytical Manual, vol. 1, Human and Environmental Media, Extraction and Cleanup, Section HE 212.02, Food and Drug Administration, Washington, D.C.
12. HAYES, W.J., JR., Ann. Rev. Pharmacol. 5, 27 (1965).
13. ROSSITER, R.J., in Neurochemistry, K.A.C. Elliott, I.H. Page, and J.H. Quastel, Eds., p 32 (1962), Charles C Thomas, Springfield, Ill.
14. McILWAIN, H., Biochemistry and the Central Nervous System, 3rd ed., pp 27, 197-198 (1966). Little, Brown, & Co., Boston.
15. Scientific Tables, 6th ed., K. Diem, Ed., pp 561, 596 (1962), Geigy, Ardsley, New York.
16. SHANKLAND, D.L., Toxicol. Appl. Pharmacol. 6, 197 (1964).
17. BAKAY, L., The Blood-Brain Barrier (1956), Charles C Thomas, Springfield, Ill.

18. DAVSON, H., Physiology of Cerebral Spinal Fluid (1967), Little, Brown, and Co., Boston.

19. LAJTHA, A., in Neurochemistry, K.A.C. Elliott, I.H. Page, and J.H. Quastel, Eds., pp 399-430 (1962), Charles C Thomas, Springfield, Ill.

20. CSERR, H.F., Fed. Proc. 26, 1024 (1967).

21. EDMUNDSON, W.F., DAVIES, J.E., NACHMAN, G.A., and ROETH, R.L., Public Health Rept. 84, 53 (1969).

22. KEANE, W.T., and ZAVON, M.R., Arch. Environ. Health, 19, 36 (1969).

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Morphological Changes in Livers of Rats Fed Polychlorinated Biphenyls

Light Microscopy and Ultrastructure

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Male and female Sherman strain rats were fed polychlorinated biphenyls Aroclor 1260 and Aroclor 1254 at 0, 20, 100, 500 and 1,000 ppm in their diet. Rats received the dietary levels for eight months. Light microscopic changes consisted of hypertrophy of the liver cells, inclusions in the cytoplasm, brown pigment in Kupffer cells, lipid accumulation, and, at the higher dietary levels, adenofibrosis. Ultrastructural changes of the livers of exposed animals consisted of an increase in smooth endoplasmic reticulum and atypical mitochondria. Lipid vacuoles were occasionally surrounded by concentric membranes. The epithelial component of adenofibrosis consisted of goblet cells and cells that resembled the epithelium which lines the bile ducts. In general, the effect of Aroclor 1254 on the liver was more pronounced than that of Aroclor 1260.

Polychlorinated biphenyls (PCBs) are widely distributed in the environment.[1] They are marketed in the United States under the trade name Aroclor followed by a four digit number with the last two digits indicating the percent of chlorine. They were first marketed in the 1930s. Jones and Alden[2] reported a case of chloracne in a worker who had been heavily exposed to a chlorinated biphenyl. Miller[3] reported pathological changes in animals exposed to a commercial chlorinated biphenyl. He studied rats, rabbits, and guinea pigs, and found liver damage in all series of experiments, as well as skin changes in the animals receiving subcutaneous injections or applications of the material to the skin. Liver damage was most pronounced in the guinea pig. The rat livers had intercellular hyaline bodies in addition to the fatty degeneration that was observed in all three species. Bennett and his co-workers[4] also reported that Aroclors affected the livers of rats. Von Oettingen[5] discusses these earlier findings in his book *"The Halogenated Hydrocarbons: Toxicity and Potential Dangers."* After the Second World War, the PCBs were increasingly used for moisture proofing, sealing, impregnation, and vapor suppression in prolonging the residual life of insecticides and as fire retardants. They can be found in synthetic resins, synthetic and natural rubber, cellulose resins, paint, varnish, wax, asphalt, and allyl starch. They have also been used in printing inks, textile dyes, and as heat transfer fluid. In 1968, an outbreak of poisoning that involved at least 600 people occurred in western Japan where rice bran oil was contaminated with Kanechlor 400, a PCB.[6] The contamination of the oil occurred because PCBs used as heat exchangers in the manufacturing process leaked into the oil through pinholes in the pipes. This incident prompted Nishizumi[7] to study the effect of PCBs on the livers of mice and monkeys. He found a large amount of acidophilic material in the cytoplasm of hepatocytes and fatty vacuoles. Using electron microscopy, he observed "myelin figures" in the cytoplasm, and an increase in smooth endoplasmic reticulum and lipid droplets.

More recently, PCBs leaked into fishmeal from a heat exchanger of a processing plant in North Carolina. The contaminated fishmeal was fed to adult chickens and breeders. The contamination was discovered when the eggs from the breeders did not hatch. Some of the chickens also exhibited symptoms of chick edema.[8] The fact that PCBs can produce chick edema has been described repeatedly.[9-11]

Contaminants may be at least partially responsible for some of these toxic effects.

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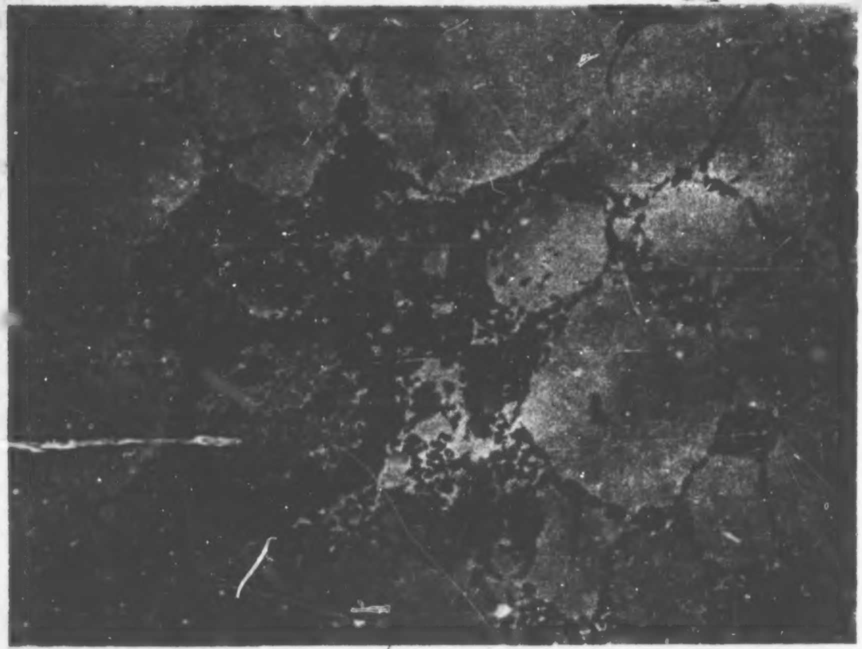


Fig 5.—Liver of turkey exposed to DMH for 21 days. Degenerated mitochondria (M), RER, and glycogen have been displaced to perinuclear region. Large lipid globules (L) with interspersed glycogen (arrow) are evident (uranyl acetate and lead citrate ×8,000).

possibility that lowered feed intake was responsible for the development of fatty metamorphosis. It is probable that toxicity occurred as a result of exposure from both the gastrointestinal and respiratory routes. This is plausible because the chemical was mixed in the feed of turkeys at the rate of 45 ml/50 lb of feed, and the experimental birds consumed 1 lb of contaminated feed per week —about 0.9 ml of DMH. However, DMH is highly volatile and the exposed feed at the bottom of the tube feeders was uncovered. Therefore, it is probable that the DMH content of the feed at the time of consumption was less than at the time of mixing of the diet, and that both the respiratory and gastrointestinal routes of exposure were involved in the induction of hepatotoxicity.

Symptoms in dogs[1] attributable to acute DMH toxicity, consisting of pulmonary distress and convulsions, were not produced in turkeys. Mild anemia, however, was produced in the turkey. The DMH did not have lathyritic activity for the turkey, as evidenced by lack of histologic alterations of the aorta.

There are numerous reports describing ultrastructural changes in hepatic cells induced by cytotoxic agents, but none is concerned with DMH. In most ultrastructural studies of hepatic fatty metamorphosis, it has been found that lipid congregates in glycogen areas and is associated with distended SER[6,7] and that there are alterations of mitochondria.[8] These alterations of hepatic cells were observed in the livers of turkeys exposed to DMH.

References

1. Witkin LB: Acute toxicity of hydrazine and some of its methylated derivatives. *AMA Arch Industr Health* 13:34-36, 1956.
2. Dimazine, in *Medical Bulletin*. New York, FMC Corp, 1961.
3. Shook BS, Coward OH: Health hazards associated with unsymmetrical dimethylhydrazine. *Ind Med Surg* 26:333-336, 1957.
4. Rosmus J, Deyl Z, Trnavsky K, et al: Experimental lathyrism. *Cesk Fysiol* 14:14-22, 1965.
5. Simpson CF, Pritchard WR, Harms RH, et al: Skeletal and cardiovascular lesions in turkeys induced by feeding β-aminopropionitrile. *Exp Mol Pathol* 1:305-320, 1962.
6. Amick CJ, Stenger RJ: Ultrastructural alterations in experimental acute hepatic fatty metamorphosis. *Lab Invest* 13:128-136, 1964.
7. Ashworth CT, Werner DS, Glass MD, et al: Spectrum of fine structural changes in hepatocellular injury due to thioacetamide. *Am J Pathol* 47:917-951, 1965.
8. Khandekar JD, Garg BD, Kovacs K: Beloxamide effects on the liver ultrastructure in rats. *Arch Pathol* 92:221-230, 1971.

Chlorinated dibenzofuran and chlorinated naphthalenes have been found as contaminants in two European commercial PCBs.[12] It is possible that other PCBs are also contaminated with these or other highly toxic substances.[13] The effect of two PCBs, Aroclor 1254 and Aroclor 1260, on Sherman strain rats, was studied in our laboratory to gain better insight into the toxicity of these commercial products. The Aroclor 1260 was from the same lot as that studied by Vos and Koeman.[11]

Materials and Methods

Sherman strain rats were used in these experiments. The animals were caged individually and weighed weekly. To determine the single dose oral LD_{50}, Aroclor 1254 and 1260 were given in peanut oil to groups of ten rats/dosage level by stomach tube as described by Gaines.[14] Survivors were observed for a minimum of 15 days after treatment. The method of Lichtfield and Wilcoxon[15] was used to calculate the LD_{50} values.

When the Aroclors were fed in the diet they were dissolved in ether and mixed with cornstarch. The ether was allowed to evaporate and the Aroclor-cornstarch mixture was mixed into increasing amounts of ground chow. In the feeding studies, 3 to 4-week-old male and female rats were distributed in groups of ten rats of each sex according to a table of random numbers. Their food consumption was measured during the second, fifth, 11th, 22nd, and 30th week of exposure to the dietary levels of 0, 100, 500, and 1,000 ppm, and at similar intervals in the groups fed 20 ppm and their controls. The rats fed 20 ppm were set up at a later date than the other dietary levels. For

Table 1.—Mean Body Weight Gain and Liver Weights of Rats Fed Aroclor 1260*

					Relative Liver Weights	
Sex	Dietary Level (ppm)	Dosage Level (mg/kg/day)	Weight Gain (gms)	Liver Weights (gms)	Fraction of Body Weight (%)	Difference From Controls
M	0	0	451	14.56	2.59	...
M	100	6.5	463	18.87	3.23	$P<.001$
M	500	32.8	398	22.01	4.25	$P<.001$
M	1,000	71.4	351	22.46	4.91	$P<.001$
F	0	0	256	11.23	3.12	...
F	100	7.2	253	12.20	3.40	$P>.10$
F	500	38.2	185	12.67	4.52	$P<.001$‡
F	1,000†	72.4	148	15.85	6.35	...
M	0	0	435	12.77	2.39	...
M	20	1.4	455	16.63	3.02	$P<.001$
F	0	0	226	11.94	3.74	...
F	20	1.6	233	12.68	3.86	$P>.50$

* In diet for eight months; ten rats/group. † Only two animals survived. ‡ For the actual liver weight $P>.05$.

Table 2.—Mean Body Weight Gain and Liver Weights of Rats Fed Aroclor 1254*

					Relative Liver Weights	
Sex	Dietary Level (ppm)	Dosage Level (mg/kg/day)	Weight Gain (gms)	Liver Weights (gms)	Fraction of Body Weight (%)	Difference From Controls
M	0	0	484	14.14	2.44	...
M	100	6.8	459	16.01	2.92	$P<.025$
M	500	36.4	315	24.48	5.95	$P<.001$
F	0	0	244	10.59	3.19	...
F	100	7.5	235	11.86	3.62	$P>.05$†
F	500	37.6	158	18.98	7.47	$P<.001$
M	0	0	453	13.96	2.53	...
M	20	1.4	439	15.38	2.90	$P<.025$
F	0	0	238	11.21	3.34	...
F	20	1.6	249	12.49	3.72	$P<.025$

* In diet for eight months; ten rats/group.
† Equal numbers of rats fed 100 ppm and 20 ppm, respectively, for same length of time did not show significant increase in actual liver weight ($P<.1$ and $>.05$); however, the increase in relative liver weight was significant $P<.01$.

this reason, a second group of controls for each sex was added to the experiments (Tables 1 and 2). All animals that were fed PCB as well as their controls were used in reproduction studies. The results of the reproduction studies will be reported separately. At autopsy, the tissues were fixed in a buffered 4% solution of formaldehyde (formalin). They were stained with hematoxylin-eosin for light microscopic study. Selected liver sections were cut on the cryostat and stained with Oil red O. Perls' stain for iron and a PAS stain were also performed on a few selected livers. Tissue from the livers of two male and two female rats at each dietary level except 20 ppm were minced and fixed in chilled 5% buffered glutaraldehyde for two hours, postfixed for two hours in 1% osmic acid (osmium tetroxide), dehydrated through changes of alcohol and propylene oxide, and embedded in resin. Sections were cut with a glass knife, stained with lead citrate and uranyl acetate, and examined with the electron microscope. In a preliminary experiment, Aroclor 1254 at the dietary level of 1,000 ppm for three months killed most of the rats. Tissue from this preliminary experiment was examined only under the light microscope.

Results

There was considerable variation, in both sexes, in the acute, oral, toxic effects of Aroclor 1260 and 1254 at various dosage levels. It was impossible to calculate LD_{50} values. The data indicated that the LD_{50} values were about 4,000 to 10,000 mg/kg for both mixtures. Autopsies of rats killed by either mixture revealed hemorrhage into the lung, stomach, and pancreas. Foci of ulceration surrounded by a severe inflammatory reaction were observed primarily in the duodenum and occasionally in the glandular part of the stomach.

When the material was fed in the diet over a period of time, porphyria was observed in a number of animals poisoned with either mixture. The liver fluoresced most consistently under the UV light. Occasionally, fluorescence of bones, serum, and urine was observed. A more detailed study of this phenomenon will be reported later.

When Aroclor 1260 was fed daily to groups of ten male and ten female rats each

Table 3.—Light Microscopic Findings in the Liver of Rats Fed Aroclor 1260*

Dietary Level (ppm)	Sex	Enlarged Hepatocytes	Inclusions in Cytoplasm	Adenofibrosis	Increased Lipid	Foamy Cytoplasm	Pigment
0	M	...	...	...	...	...	...
20	M	10	9	...	...	3	...
100	M	10	9	...	10	...	...
500	M	10	4	...	10	7	1
1,000	M	10	6	2	10	9	2
0	F	...	...	...	...	...	...
20	F	3	...	...	...	...	...
100	F	7	2	1	10	...	1
500†	F	9	5	1	9	8	6
1,000‡	F	6	...	4	7	4	2

* In their diet for eight months; ten rats/group. † Only nine animals were studied. ‡ Only seven animals were studied.

Table 4.—Light Microscopic Findings in the Liver of Rats Fed Aroclor 1254*

Dietary Level (ppm)	Sex	Enlarged Hepatocytes	Inclusions in Cytoplasm	Adenofibrosis	Increased Lipid	Foamy Cytoplasm	Pigment
0	M	...	...	...	...	...	...
20	M	7	1	...	...	1	...
100	M	10	7	1	10	10	3
500	M	10	2	10	10	4	2
0	F	...	...	...	...	...	...
20	F	...	...	...	...	...	1
100	F	10	7	7	10	9	10
500†	F	9	2	9	9	2	7

* In their diet for eight months; ten rats/group.
† Only nine tested since one rat that died showed too much autolysis.

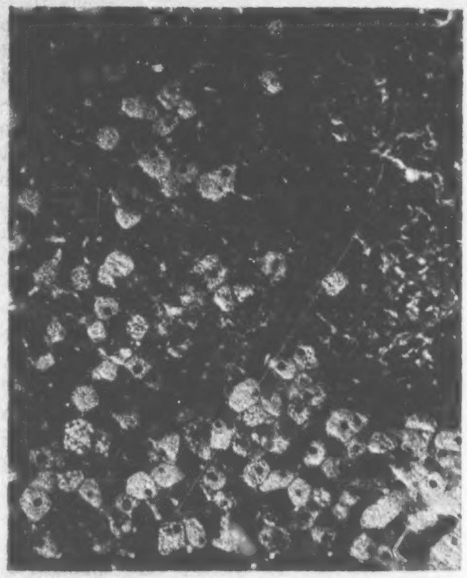


Fig 1.—Liver section of male rat fed 500 ppm Aroclor 1260 for eight months. Note foamy cytoplasm of many of hepatocytes (hematoxylin-eosin ×125).

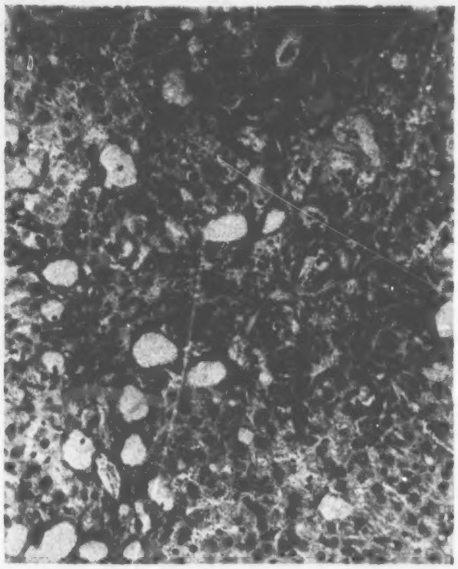
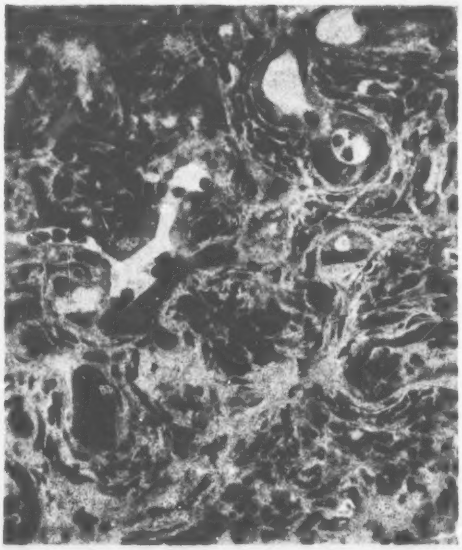


Fig 2.—Section of liver from male rat fed 1,000 ppm Aroclor 1254 for eight months illustrating adenofibrosis. Note area of fibrosis which surrounds ductules of glandular cells. Cellular debris, polymorphonuclear leukocytes, or mucus can be observed in lumen of ductules (hematoxylin-eosin ×125).

at dietary levels of 0, 20, 100, 500, and 1,000 ppm for eight months, none of the control rats and none of the male rats fed the mixture died. One female fed 100 ppm died after six months exposure and two females fed 500 ppm died after one and two months exposure. Eight females fed 1,000 ppm died within two to six months after onset of the experiment. The rats fed 500 and 1,000 ppm Aroclor 1260 in their diet gained less weight than the controls. At autopsy, the livers of all male rats fed Aroclor 1260 weighed significantly more than the livers of the controls (Table 1). The livers of the female rats were larger also but a significant weight difference was not observed, except that at 500 ppm the percent of body weight of the livers was increased as a result of the reduced weight gain of these animals (Table 1).

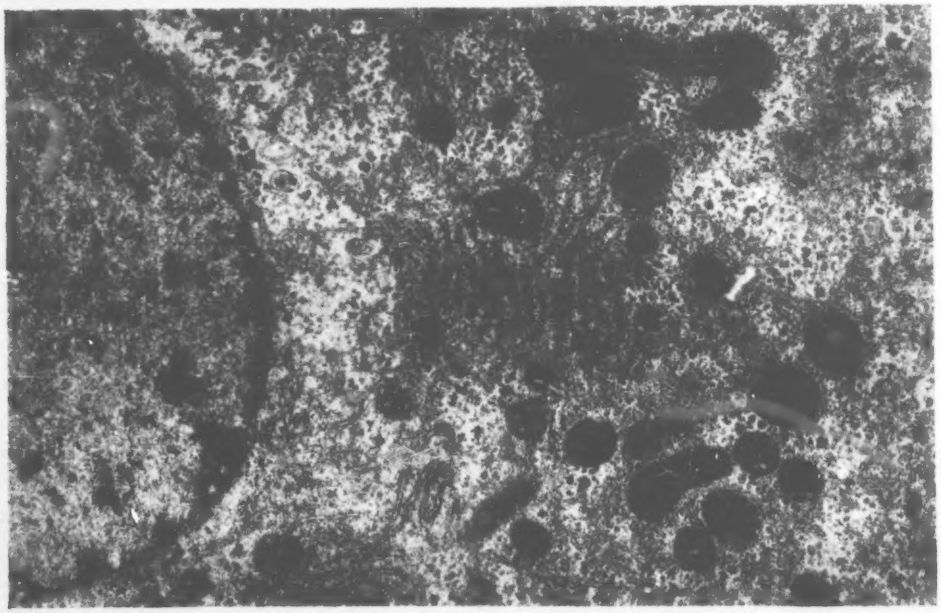
Grossly, the livers of the exposed animals were soft and often yellowish brown or dark olive. Grayish-white, firm, glistening areas, measuring up to 0.5 cm in diameter, were seen on cross section in a number of livers. The dark livers showed pink-orange fluorescence under the UV light.

Fig 3.—Area of adenofibrosis as in Fig ~~2~~ 3 from female rat fed 500 ppm Aroclor 1254 for eight months. Area was examined under electron microscope (Fig 8-10) (toluidine blue ×300).



The incidence of the various light microscopic findings of the livers are given in Table 3. They consisted of hypertrophy of the individual liver cells. Often the nuclei were hyperchromatic and showed pleomorphism, and occasional mitotic figures were observed. The cytoplasm had either large vacuoles or appeared foamy (Fig 1). Eosinophil inclusions identical to those described in the liver of rats that have been exposed to DDT,[16] mirex,[17] or dieldrin[18] were also observed. The Kupffer cells and perivascular macrophages of some livers contained a brown pigment. Frozen sections of livers containing the brown pigment fluoresced pink under UV light which is consistent with the presence of porphyrin. Some of the pigment showed a positive staining reaction for iron with Perls' stain as well. In addition to these findings, some livers also exhibited focal areas of fibrosis which contained glandular structures (Fig 2 and 3) that either formed strands or dilated cystic ducts that were filled with cellular debris (polymorphonuclear leukocytes) or a mucus-like PAS-positive substance. The glandular cells had a pale, scanty, basophilic cytoplasm and usually a hypochromatic nucleus. The areas of adenofibrosis corresponded to the grayish-white, glistening areas described grossly. The glandular epithelium appeared stratified in some areas. Electron microscopic study revealed (Fig 4 to 10) an increase in smooth endoplasmic reticulum (Fig 5) in the hepatocytes of all but the control rats (Fig 4). Many large and small lipid vacuoles and occasional atypical mitochondria were also seen. At the dietary level of 100 ppm, dark and light cells, dilated bile ducts, and granular cytoplasmic inclusions were additional findings (Fig 6). At the dietary level of 500 and 1,000 ppm, concentrically arranged membranes which surrounded lipid vacuoles were also observed. They have been previously described in livers of rats fed DDT and dieldrin.[18] The cytoplasm of many cells had a loosened-up, "moth eaten" appearance (Fig 7), with occasional vacuoles containing a few strands of electron-dense material. These cells corresponded to the hepatocytes with foamy cytoplasm observed under the light microscope. Widening of intercellular spaces, occasional phagocytized red blood cells, and an increase in collagen were observed. The nodular, grayish-white areas (Fig 8) that represented

Fig 4.—Section from normal male rat liver. N, nucleus; M, mitochondria; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum (lead citrate, uranyl acetate ×13,490).



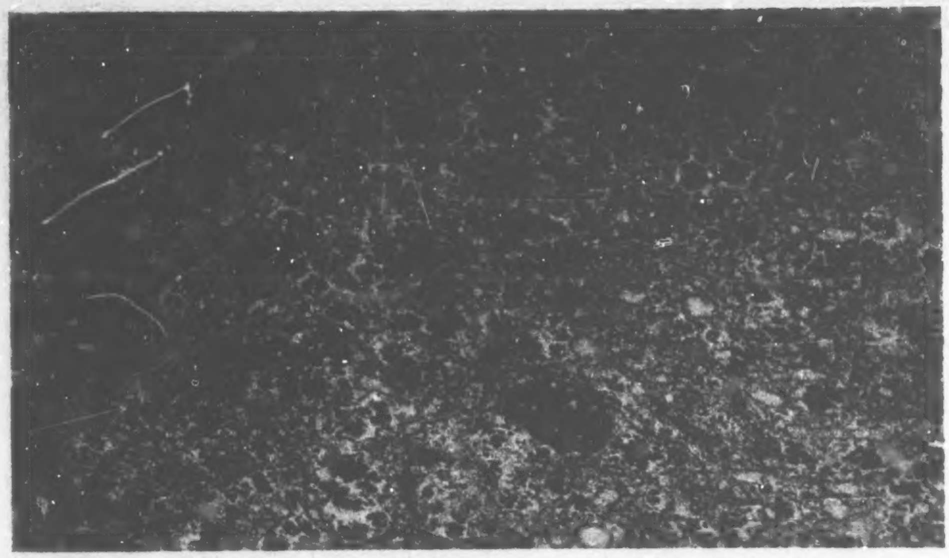


Fig 5.—Section from male rat liver fed 100 ppm Aroclor 1260 for eight months. Note large amount of smooth endoplasmic reticulum. SER, smooth endoplasmic reticulum; M, mitochondria; N, nucleus (lead citrate, uranyl acetate ×34,200).

Fig 6.—Section from liver of female rat fed 500 ppm Aroclor 1260 for eight months. Note small and large vacuoles within cytoplasm, some filled with lipid and others with strands of dark granular material (arrows). V, vacuoles; M, mitochondria; CB, cell border; BC, bile canaliculus; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum (lead citrate, uranyl acetate ×13,490).

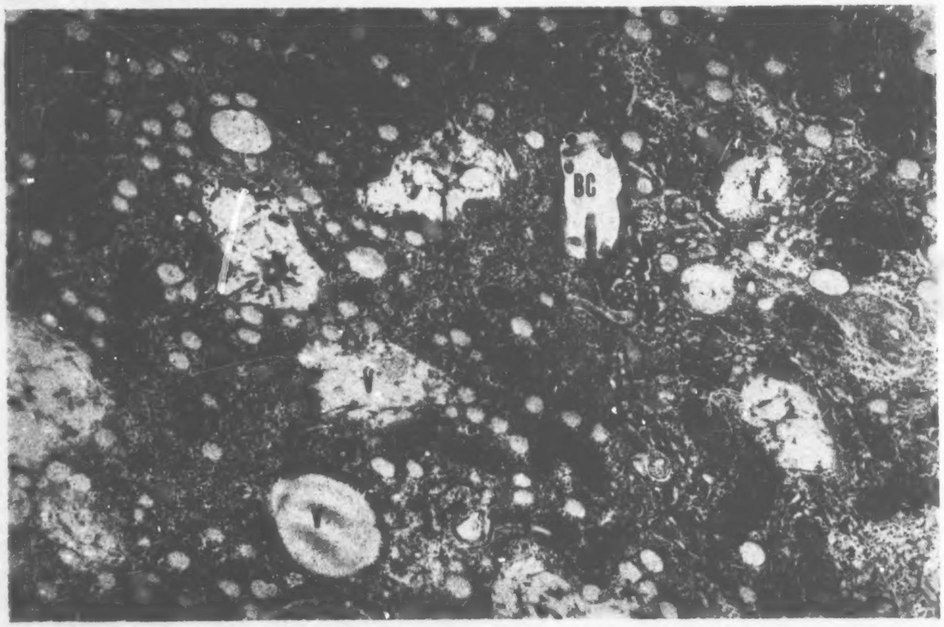
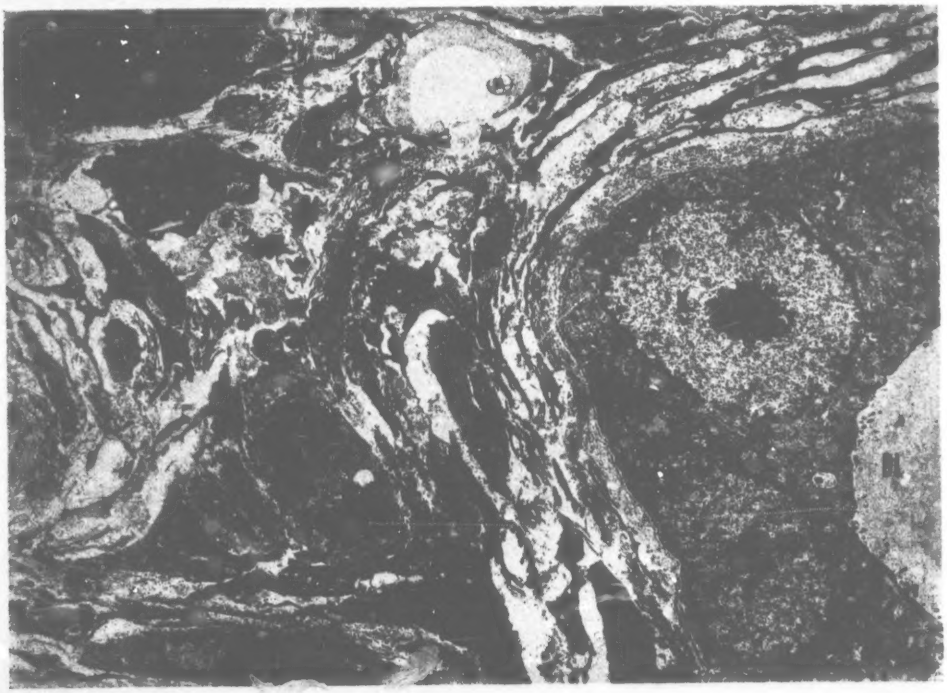


Fig 7.—Section from liver of male rat fed 1,000 ppm Aroclor 1260 for eight months. Note "moth-eaten" appearance of the cytoplasm (lead citrate, uranyl acetate ×13,490).

Fig 8.—Section from liver of female rat fed Aroclor 1254 for eight months. Note large amount of collagen, fibroblasts, and epithelial cells lining a ductule. C, collagen; FB, fibroblast; N, nucleus; EC, epithelial cell (arrow); DL, lumen of ductule (lead citrate, uranyl acetate ×5,320).

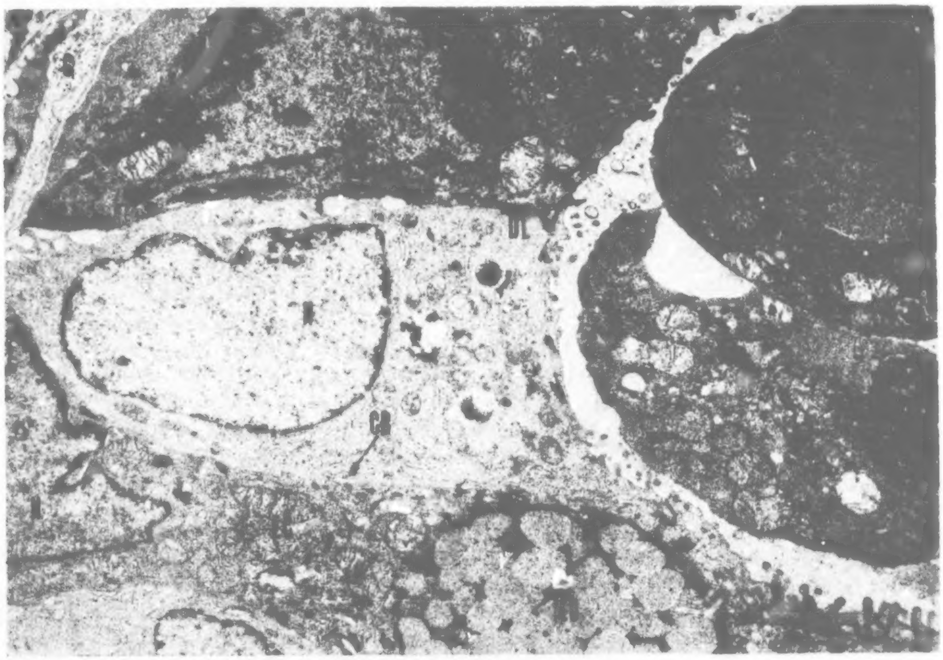


extensive foci of adenofibrosis consisted of fibroblasts and a great deal of collagen that surrounded rosettes of epithelial cells. These cells were cuboidal or columnar in shape. The free surface was lined by microvilli, and cells contained large, pale nuclei. The cytoplasm was granular, with many ribosomes and a few endoplasmic membranes. Some of these liver duct cells contained a great deal of mucus and resembled goblet cells similar to those observed in the intestinal tract (Fig 9). The cytoplasm contained tonofilaments and, particularly near the duct lumen, the boundaries between adjacent cells were marked by prominent terminal bars (Fig 10).

In a preliminary study in which 1,000 ppm Aroclor 1254 was fed in the diet to ten male and ten female rats, five of the males and eight of the females died. At autopsy of the exposed animals, the livers were pale and enlarged and contained firm, grayish-white, glistening areas. One male rat that died after 49 days exposure showed only slight enlargement of the liver cells. The hepatocytes of all the other exposed rats were enlarged, vacuolated often with hyperchromatic—sometimes enlarged and sometimes pyknotic—nuclei which occasionally displayed mitotic figures. Varying degrees of fibrosis around the bile ducts were observed. Hyperplasia and proliferation of glandular cells simulating bile ducts were also seen in these areas. In some areas, the sinusoids were dilated and contained cellular debris, lined by small "compressed" hepatocytes. A brown pigment was seen within the macrophages. A few livers stained with Oil red O showed a large amount of red staining (lipid) material. The animals that died not only had liver lesions but also suffered from severe pulmonary congestion and, in a few instances, from massive hemorrhages into the soft tissue or the peritoneal cavity.

When 500, 100, 20, and 0 ppm Aroclor 1254 were fed to the rats for eight months, two males and one female rat died at 500 ppm, but none died at the lower dietary levels. The rats fed 500 ppm Aroclor 1254 gained less weight than the controls. The

Fig 9.—Section from same liver as Fig 8. Note goblet cell containing mucus and degenerated cells within lumen of ductule. C, collagen; EC, epithelial cell; N, nucleus; CB, cell border (arrow); DL, lumen of ductule (arrow); MU, mucus goblet cell (lead citrate, uranyl acetate ×11,400).



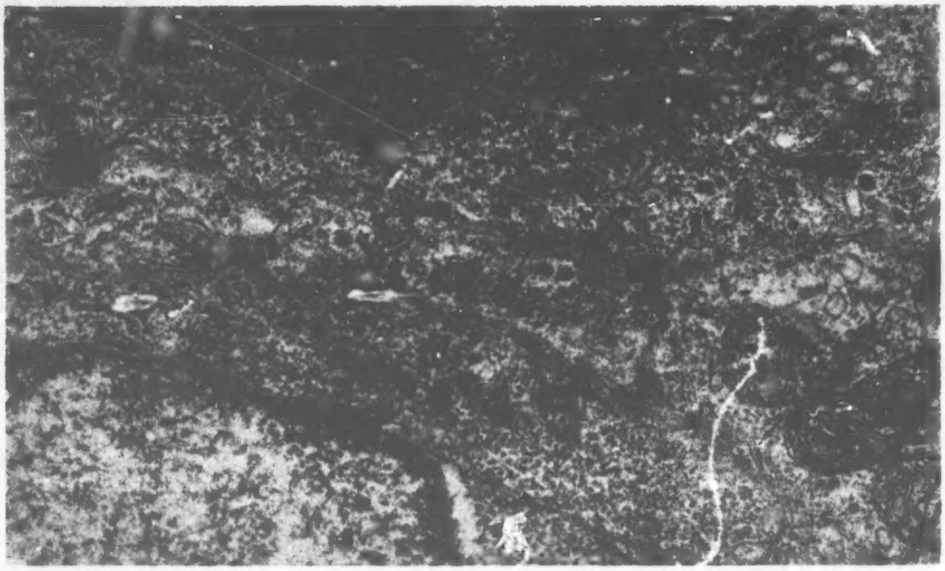


Fig 10.—Higher magnification of two epithelial cells from Fig 9 showing T, tonofilaments; D, desmosomes; N, nucleus; M, mitochondria; CB, cell border (lead citrate, uranyl acetate ×34,200).

livers of the exposed groups were larger than those of the controls (Table 2). This was significant in all but the female rats fed 100 ppm. At autopsy, the livers of the exposed animals, particularly at the higher dietary levels, were soft, yellowish-brown, or dark olive. Firm, grayish-white, glistening areas were seen, especially in those fed higher PCB levels. Occasionally, the liver surface was nodular. These livers also fluoresced when exposed to UV light. The light microscopic findings are given in Table 4. The observed changes in the livers of exposed rats were quite similar to those described for Aroclor 1260, except that all male rats at 500 ppm and one male rat at 100 ppm had areas of adenofibrosis in their liver, while none of the males demonstrated this at the respective dietary levels of Aroclor 1260. In the females, the incidence of adenofibrosis was much higher at the same dietary levels of Aroclor 1254 than in those fed Aroclor 1260. The electron microscopic examination of the different liver changes summarized in Table 4 corresponded to those already described for Aroclor 1260.

The main difference between the two compounds was the much higher incidence of adenofibrosis at a lower dietary level of Aroclor 1254. When fed over a period of time, Aroclor 1260 seemed to be more toxic to female rats. Such a sex difference could not be established for Aroclor 1254.

Comment

Aroclor 1260 and 1254 have a definite effect on the liver. This effect is more pronounced with Aroclor 1254 when all morphologic changes of equivalent dietary levels of Aroclor 1254 and 1260 are compared. All dietary levels tested so far showed an increase in the size of the individual liver cells and often an increase in the liver weights. Accumulation of fat was observed at 100 ppm, and it was very pronounced at the higher dietary levels. The incidence of cytoplasmic inclusions was higher at the lower dietary levels. This is probably due to the fact that at the higher dietary levels most of the cytoplasmic components have been replaced by lipids. The pigment observed in the Kupffer cells showed a positive prussian blue reaction and represented, at least in part, hemosiderin. The fluorescence of the livers under UV light was caused by the presence of porphyrin, which resulted in the dark discoloration of the liver.

An additional finding in the livers was the presence of grayish-white, glistening areas which microscopically corresponded to adenofibrosis. This lesion consisted of glandular, often mucus-producing, epithelial cells forming ductules. They were surrounded by fibrosis. Synonyms for this lesion are cholangiofibrosis, bile duct proliferation, bile duct adenomatosis, and fibroadenoma. Stewart and Snell[19] give an excellent description of adenofibrosis of the liver of rats. The authors concluded that "adenofibrosis is a contracting lesion built up by the incorporation and transformation of normal (hepatic cords) and pathologic (pseudotubules) components of the surrounding liver. Thus far there is no convincing evidence that adenofibrosis is a precancerous lesion." Adenofibrosis, for instance, has been described in livers of rats fed butter yellow (p-dimethylaminoazobenzene).[20] Recently, Reuber[21] fed hamsters 2-acetamidofluorene or 2-diacetamidofluorene and produced adenocarcinomas. He thought that some of these carcinomas originated from bile ducts. The author interpreted adenofibrosis as cholangiofibrosis, suggesting that adenofibrosis in the rat was a precancerous lesion and was identical to the lesions he observed in hamsters which, in his opinion, also represented a precancerous lesion. He postulated that the observed cholangiocarcinomas developed from the adenofibrosis. Glaser and his colleagues[22] produced a highly malignant transplantable tumor, derived from the liver of a hamster fed 2-acetylaminofluorene, that did not appear to be malignant in the donor but showed changes similar to the adenofibrosis described in this paper. It is possible that the malignant lesion was not simply derived from the adenofibrosis-type lesion but from cells that already manifested irreversible preneoplastic changes that were not microscopically detectable.

Miller[8] found fatty livers, liver necrosis, and inclusions in the cytoplasm of the hepatocytes in rats that had been exposed to a biphenyl with 42% chlorination. He did not mention adenofibrosis in his report. However, he studied a different biphenyl and his animals were exposed to the chemical for much shorter periods of time. Nishizumi[7] studied the effect of a biphenyl with 48% chlorination in monkeys and mice. The longest exposure time was 26 weeks. He described enlargement of the liver, fatty changes, granular cytoplasm of the hepatocytes, increase in size of Kupffer cells and hepatocytes, and a brown pigment in some of these cells, but did not observe adenofibrosis. At some stages of exposure he observed, with the electron microscope, an increase in the smooth endoplasmic reticulum, variation in the appearance of mitochondria, and an increase in the number of microbodies, as well as "myelin figures" which seem to be similar to the concentrically arranged membranes that have been described in rats after exposure to chlorinated hydrocarbons.[18] The increase in liver weights and the observation of inclusions in the cytoplasm has been reported for several persistent insecticides.[16-18] To our knowledge adenofibrosis has not been produced with chemicals such as DDT, dieldrin, pyrethrum, piperonyl butoxide, and mirex. Unlike Nishizumi's findings,[7] an increase in the number of microbodies was not observed in the rats fed PCBs in this study.

The increase in smooth endoplasmic reticulum has also been observed with other chemicals, and it is not surprising that chlorinated biphenyls induce microsomal enzymes.[23-25]

Study of the adenofibrosis under the electron microscope shows the epithelial component of the lesion consisting of two types of cells—goblet cells and epithelial cells with granular cytoplasm, tonofilaments, and desmosomes. The latter cells resemble the epithelial cells lining the bile ducts rather than hepatocytes. The epithelial cells are reminiscent of "oval" cells which are thought to represent ductular cells. However, the oval cells are smaller and have a hyperchromatic nucleus.[26] Whether they develop originally from bile duct epithelium or from other cells in the liver needs further investigation. The fact that adenofibrosis has not been observed in previous feeding studies with PCBs may have a variety of reasons such as the length of exposure, the animal species or rat strain, and the composition of PCB given to the rats. Contamination of PCBs with traces of other chemicals such as chlorinated dibenzodioxins, chlorinated dibenzofurans, or chlorinated naphthalenes may also be responsible. Vos and Koeman[11] checked Aroclor 1260 of the same lot and also did not demonstrate any contaminants. However, preliminary chemical analyses of this lot of Aroclor 1254 suggest possible contamination with a chlorinated dibenzofuran. This is still under investigation.[27]

The significance of adenofibrosis will be further investigated in order to establish whether it is an irreversible lesion and whether it is transplantable. From experience with DDT and other chlorinated hydrocarbons, it can be deduced that the liver enlargement, as expressed by hypertrophy of the liver cells and the observation of inclusions in the cytoplasm, is reversible after cessation of exposure.

The observed porphyria, which was also noted by Vos and Koeman,[11] and the effect of PCBs on the liver, particularly the adenofibrosis, make it necessary to reevaluate our present concept of the toxicity of PCBs. Additional information is also needed on the interaction of PCBs with substances such as alcohol, chlorinated hydrocarbons, sex hormones, lead, and, of course, the various contaminants that may or may not be present in the PCBs. The quality of the diet may have an impact on the effect of PCBs. The findings described in this paper support the conclusion of Lichtenstein et al[28]—that the toxicity of the PCBs decreases as the level of chlorination increases.

Richard L. Moore assisted with the animal tests. Estelle C. Gray did the statistical analysis. Linda W. Anderson and Annie R. Alford prepared the tissues.

Aroclor 1254 (lot No. AK-38) and Aroclor 1260 (lot No. AK-3) were provided gratis by Monsanto Chemical Co, St. Louis, Mo.

References

1. Risebrough RW, Rieche P, Peakall DB, et al: Polychlorinated biphenyls in the global ecosystem. *Nature* 220:1,078-1,102, 1968.

2. Jones JW, Alden HS: An acneform dermatergosis. *Arch Derm Syph* 33:1,022-1,034, 1936.

3. Miller JW: Pathologic changes in animals exposed to a commercial chlorinated diphenyl. *Public Health Rep* 59:1,085-1,093, 1944.

4. Bennett GA, Drinker CK, Warren MF: Morphological changes in the livers of rats resulting from exposure to certain chlorinated hydrocarbons. *J Indust Hyg Toxicol* 20:97-123, 1938.

5. Von Oettingen WF: *The Halogenated Hydrocarbons: Toxicity and Potential Dangers*, publication 414. US Public Health Service, 1955, pp 306-307.

6. Kuratsune M, Morikawa Y, Hirohata T, et al: An epidemiologic study on "yusho" or chlorobiphenyls poisoning. *Acta Med* (*Fukuoka*) 60:513-532, 1969.

7. Nishizumi M: Light and electron microscope study of chlorobiphenyl poisoning: In mouse and monkey liver. *Arch Environ Health* 21:620-632, 1970.

8. Pichirallo J: PCB's: Leaks of toxic substances raises issue of effects regulations. *Science* 173:899-902, 1971.

9. McCune EL, Savage JE, O'Dell BL: Hydropericardium and ascites in chicks fed a chlorinated hydrocarbon. *Poult Sci* 41:295-299, 1962.

10. Flick DF, O'Dell RG, Childs VA: Studies of the chick edema disease: III. Similarity of symptoms produced by feeding chlorinated biphenyls. *Poult Sci* 44:1,460-1,465, 1965.

11. Vos JG, Koeman JH: Comparative toxicologic study with polychlorinated biphenyls in chickens with special reference to porphyria, edema formation, liver necrosis and tissue residues. *Toxic Appl Pharmacol* 17:656-668, 1970.

12. Vos JG, Koeman JH, van der Maas HL, et al: Identification and toxicological evaluation of chlorinated dibenzofuran and chlorinated naphthalene in two commercial polychlorinated biphenyls. *Food Cosmet Toxic* 8:625-635, 1970.

13. Kimbrough RD: Toxicity of chlorinated hydrocarbons and related compounds including chlorinated dibenzodioxins and chlorinated dibenzofurans: A review. *Arch Environ Health* 25:125-131, 1972.

14. Gaines TB: The acute toxicity of pesticides to rats. *Toxic Appl Pharmacol* 2:88-99, 1960.

15. Lichtfield JJ, Wilcoxon F: A simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther* 96:99-133, 1949.

16 Kimbrough RD, Gaines TB, Hayes WJ: Combined effect of DDT, pyrethrum, and piperonyl butoxide on rat liver. *Arch Environ Health* 16:333-341, 1968.

17. Gaines TB, Kimbrough RD: Oral toxicity of mirex in adult and suckling rats: With notes on the ultrastructure of liver changes. *Arch Environ Health* 21:7-14, 1970.

18. Kimbrough RD, Gaines TB, Linder RE: The ultrastructure of livers of rats fed DDT and dieldrin. *Arch Environ Health* 22:460-467, 1971.

19. Stewart HL, Snell KC: The histopathology of experimental tumors of the liver of the rat: A critical review of the histopathogenesis. *Acta Unio Internationalis Contra Cancrum* 13:770-803, 1957.

20. Edwards JE, White J: Pathologic changes with special reference to pigmentation and classification of hepatic tumors in rats fed p-dimethylaminoazobenzene (butter yellow). *J Nat Cancer Inst* 2:157-183, 1941.

21. Reuber MD: Histogenesis of cholangiofibrosis and well differentiated cholangiocarcinoma in Syrian hamsters given 2-acetoamidofluorene or 2-diacetoamidofluorene. *Gann* 59:239-246, 1968.

22. Glaser OG, Newberne PM, Gebliks J, et al: Transplantable malignant cholangiocarcinoma from hamster liver. *Arch Environ Health* 23:137-141, 1971.

23. Street JC, Urry FM, Wagstaff DJ, et al: Comparative effects of polychlorinated biphenyls and organochlorine pesticides in induction of hepatic microsomal enzymes. Read before the 158th meeting of the American Chemical Society, Pesticide Chemistry Division, New York, 1969.

24. Villeneuve DC, Grant DL, Phillips WEJ, et al: Effects of PCB administration on microsomal enzyme activity in pregnant rabbits. *Bull Environ Cont Tox* 6:120-128, 1971.

25. Litterst CL, Farber TM, Baker AM, et al: Effect of Aroclors on hepatic microsomal enzymes in the rat, abstracted. *The Pharmacologist* 13:288, 1971.

26. Grisham JW, Hartcroft WS: Morphologic identification by electron microscopy of "oval" cells in experimental hepatic degeneration. *Lab Invest* 10:317-332, 1961.

27. Curley A, Burse VW, Jennings R: Metabolite or contaminant of Aroclor 1254 found in rat urine. Read before the national meeting of the American Chemical Society, Division of Pesticide Chemistry, Boston, 1972.

28. Lichtenstein EP, Schulz KR, Fuhremann TW, et al: Biological interaction between plasticizers and insecticides. *J Econ Entom* 62:761-766, 1969.